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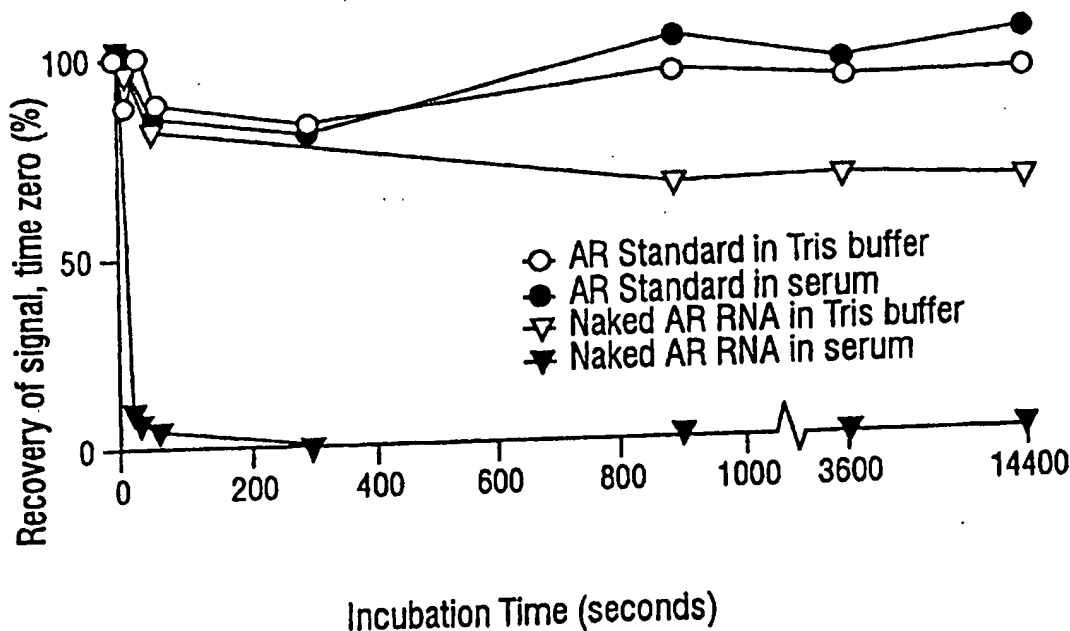
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(54) Title: RIBONUCLEASE RESISTANT RNA PREPARATION AND UTILIZATION



(57) Abstract

The present invention relates to nuclease resistant nucleic acids in general and ribonuclease resistant RNAs in particular. Methods of making and using such nucleic acids are disclosed.

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DESCRIPTION**RIBONUCLEASE RESISTANT RNA PREPARATION AND
UTILIZATION**

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BACKGROUND OF THE INVENTION

This is a continuation-in-part of co-pending application Serial No. 08/675,153, filed July 3, 1996, and a continuation-in-part of co-pending provisional application Serial No. 60/021,145, filed July 3, 1996.

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In the last few years, diagnostic assays and assays for specific mRNA species have been developed based on the detection of specific nucleic acid sequences. These assays depend on such technologies as RT-PCR™ (Mulder, 1994), isothermal amplification (NASBA) (Van Gemen, 1994), and branched chain DNA (Pachl, 1995). Many of these assays have been adapted to determine the absolute concentration of a specific RNA species. These absolute quantification assays require the use of an RNA standard of which the precise amount has been previously determined. These RNA standards are usually synthesized by *in vitro* transcription or are the infectious agents themselves. The RNA is purified and then quantified by several different methods, such as absorbance at OD₂₆₀, phosphate analysis, hyperchromicity or isotopic tracer analysis (Collins, 1995).

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Quantifying virus RNA sequences in plasma is an important tool for assessing the viral load in patients with, for example, Human Immunodeficiency Virus (HIV), Hepatitis C Virus (HCV), and other viruses such as HTLV-1, HTLV-2, hepatitis G, enterovirus, dengue fever virus, and rabies. Viral load is a measure of the total quantity of viral particles within a given patient at one point in time. In chronic infections viral load is a function of a highly dynamic equilibrium of viral replication and immune-mediated host clearance. The benefits of determining viral load include the ability to: 1) assess the degree of viral replication at the time of diagnosis - an estimate having prognostic implications, 2) monitor the effect of antiviral medications early in the disease course, and 3) quickly assess the effects of changing antiviral medications.

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Presently, the most sensitive method available for HIV quantification in plasma employs PCR™. There are 4 major steps involved in PCR™ analysis of HIV: 1) Sample

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preparation, 2) Reverse transcription, 3) Amplification, and 4) Detection. Variability in any of these steps will affect the final result. An accurate quantitative assay requires that each step is strongly controlled for variation. In the more rigorous PCR™ assay formats, a naked RNA standard is added to the denaturant just prior to the isolation of the viral RNA from plasma (Mulder, 1994). A less precise method is to add the standard to the viral RNA after it has been purified (Piatak, 1993). It is important that the RNA standards are precisely calibrated and that they withstand the rigors of the assay procedures.

There is a need for ribonuclease resistant RNA standards. RNA is susceptible to environmental ribonucleases. Producing ribonuclease-free reagents is non-trivial. A danger in using naked RNA as a standard for quantification is its susceptibility to ribonuclease digestion. Compromised standards generate inaccurate values. This problem can be compounded in clinical laboratory settings where the personnel are not usually trained in RNA handling. These factors introduce doubt as to the validity of the data generated.

Naked RNA standards are very susceptible to ribonuclease digestion. Some RNA based assays have been formatted so that users access an RNA standard tube only once and then discard it to minimize the possibility of contaminating the RNA standard with ribonucleases. However, the standards are aliquoted into microfuge tubes which are not guaranteed to be ribonuclease-free introducing another potential source for contamination. As well, there is a short period of time during which the RNA is exposed to a pipette tip before it is placed in the denaturing solution. If the pipette tip is contaminated with ribonuclease then the RNA standard will be degraded and the assay compromised. Another disadvantage of using naked RNA standards are that they must be stored frozen. In the branched DNA HIV assay formatted by Chiron Corp., the potential for RNA degradation is so risky that their assays include single stranded DNA instead of RNA for their standard (Pachl, 1995). The DNA is calibrated against RNA. The DNA standard is much less likely to be degraded. Thus, there is a need for RNA standards which are resistant to ribonucleases and in which there is no doubt about the integrity of the standard. These standards would also be more convenient if they did not need to be stored frozen so that they could be used immediately, no thawing required.

Those of skill know how to bring about chemical alteration of RNA. Such alterations can be made to nucleotides prior to their incorporation into RNA or to RNA after it has been formed. Ribose modification (Piecken 1991) and phosphate modification (Black, 1972) have been shown to enhance RNA stability in the presence of nucleases. Modifications of the 2' hydroxyl and internucleotide phosphate confers nuclease resistance by altering chemical groups that are necessary for the degradation mechanism employed by ribonucleases (Heidenreich, 1993). While such chemical modification can confer ribonuclease resistance, there is no known suggestion in the art that such ribonuclease resistant structures could be useful as RNA standards.

RNA bacteriophages have long been used as model systems to study the mechanisms of RNA replication and translation. The RNA genome within RNA bacteriophages is resistant to ribonuclease digestion due to the protein coat of the bacteriophage. Bacteriophage are simple to grow and purify, and the genomic RNA is easy to purify from the bacteriophages. These bacteriophages are classified into subgroups based on serotyping. Serologically, there are four subclasses of bacteriophage, while genetically, there are two major subclasses, A and B (Stockley, 1994; Witherell, 1991). Bacteriophage MS2/R17 (serological group I) have been studied extensively. Other well-studied RNA bacteriophages include GA (group II), Q-beta (group III), and SP (group IV). The RNA bacteriophages only infect the male strains of *Escherichia coli*, that is, those which harbor the F' plasmid and produce an F pilus for conjugation.

The MS2 bacteriophage is an icosahedral structure, 275 Å in diameter, and lacks a tail or any other obvious surface appendage (Stockley, 1994). The bacteriophage has large holes at both the 5- and 3-fold axes which might be the exit points of the RNA during bacterial infection. The MS2 bacteriophage consists of 180 units of the bacteriophage Coat Protein (~14 kDa) which encapsidate the bacteriophage genome (see reviews, Stockley, 1994; Witherell, 1991). The MS2 RNA genome is a single strand encoding the (+) sense of 3569 nucleotides. The genes are organized from the 5' end as follows: the Maturase or A protein, the bacteriophage Coat Protein, a 75 amino acid Lysis Protein, and a Replicase subunit. The Lysis gene overlaps the Coat Protein gene and the Replicase gene and is translated in the +1 reading frame of the Coat Protein.

Each bacteriophage particle has a single copy of Maturase which is required for interacting with the F pilus and thus mediating bacterial infection.

Packaging of the RNA genome by Coat Protein is initiated by the binding of a dimer of Coat Protein to a specific stem-loop region (the Operator or "*pac*" site) of the RNA genome located 5' to the bacteriophage Replicase gene. This binding event appears to trigger the complete encapsidation process. The sequence of the Operator is not as critical as the stem-loop structure. The Operator consists of ~21 nucleotides and only two of these residues must be absolutely conserved for Coat Protein binding.

The viral Maturase protein interacts with the bacteriophage genomic RNA at a minimum of two sites in the genome (Shiba, 1981). It is evidently not required for packaging. However, its presence in the bacteriophage particle is required to preserve the integrity of the genomic RNA against ribonuclease digestion (Argetsinger, 1966; Heisenberg, 1966).

Attempts to produce a viable, infectious recombinant RNA (reRNA) bacteriophage have been unsuccessful. The bacteriophage are very efficient at deleting heterologous sequences and the fidelity of the Replicase is poor such that point mutations occur at the rate of $\sim 1 \times 10^{-4}$.

Pickett and Peabody (1993) performed studies in which a non-bacteriophage RNA was encapsidated by MS2 Coat Protein. Their apparent goal was to determine if the 21 nucleotide Operator (*pac* site) would confer MS2-specific packageability to non-bacteriophage RNA *in vivo*. *E. coli* was co-transformed with two plasmids: one encoding MS2 Coat Protein and the other encoding β -galactosidase (*lacZ*). The *lacZ* gene was modified such that it had the MS2 Operator sequence cloned upstream of it. The *E. coli* were induced such that the Operator-*lacZ* hybrid RNA was co-expressed with the MS2 Coat Protein. The Coat Protein dimer bound to the Operator, triggering the encapsidation of the *lacZ* RNA to form "virus-like particles". The virus-like particles were purified by a CsCl gradient. The buoyant density of these virus-like particles had a much greater density distribution than did the wild-type MS2 bacteriophage. The MS2 banded tightly at 1.45 g/cc whereas the virus-like particles ranged in density from 1.3 to 1.45 g/cc, suggesting substantial heterogeneity in the RNA content of the virus-like

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particles. In other words, the Pickett and Peabody virus-like particles were packaging different lengths of RNA and/or different species of RNAs.

The results of the Pickett and Peabody work were not as expected. The *lacZ* RNA purified from these virus-like particles was degraded to a major species of ~500 bases as opposed to the expected full length 3000 bases. This 500 base RNA was only detectable by the sensitive Northern blotting procedure. The authors did not know if the degradation occurred before or after encapsidation, but suggested that these viral-like particles may be sensitive to ribonuclease digestion. It was found that the majority of the RNA packaged was actually 2 species, 1800 bases and 200 bases in size. These two RNA fragments were easily detected after gel electrophoresis and methylene blue staining. The 500 base Operator-*lacZ* RNA fragment was not visible by methylene blue staining. It was only detected by Northern blotting using a *lacZ* probe. These authors concluded that the 0.2 and 1.8 kb RNAs were derived from *E. coli* pre-16S rRNA. The host *E. coli* RNA was packaged in preference to the Operator-*lacZ* RNA indicating that the specificity of the Pickett and Peabody bacteriophage packaging system was poor.

In other studies, Pickett and Peabody modified the packaging of the Operator-*lacZ* RNA by changing the ratios of the Coat Protein and Operator-*lacZ* RNA produced in *E. coli*. By increasing the concentration of the Operator-*lacZ* RNA and decreasing the concentration of the Coat Protein, they were able to encapsidate mainly the Operator-*lacZ* RNA and no detectable pre-16S rRNA. These results suggested that the original Pickett and Peabody packaging strategy suffered in specificity because they were unable to reach and maintain the appropriate molar ratio of Coat Protein to Operator-*lacZ* RNA optimal for packaging the target RNA. Even in the second set of packaging studies, the concentrations of the Coat Protein and Operator-*lacZ* RNA were only coarsely adjusted. The Pickett and Peabody system had no feedback mechanism to maintain the optimal ratio of Coat Protein to Operator-*lacZ* RNA for packaging.

In their second set of packaging studies, Pickett and Peabody did not characterize the RNA that was packaged with the modified procedure. The RNA was not purified from the virus-like particles and assessed by, for example, gel electrophoresis. Furthermore, the virus-like particles in this study or the previous study were not characterized for their ability to protect the encapsidated *lacZ* RNA from ribonucleases.

There was no discussion as to the yield of virus-like particles or Operator-*lacZ* RNA obtained from the Pickett and Peabody studies.

Currently, there are two major methods for the synthesis of RNA species of a specific sequence: chemical synthesis and *in vitro* transcription. Although the chemical synthesis of RNA can produce very pure product, it is both expensive and it is limited to synthesizing oligonucleotides not much longer than 30 bases. Chemical synthesis is most suitable for generating antisense RNA oligonucleotides, which are generally 15 to 30 bases in length. However, most of the applications for RNA, such as probe synthesis and *in vitro* and *in vivo* translation, require longer RNA products, in the range of 100 bases to several kilobases.

RNA synthesis by *in vitro* transcription became a practical method as developed by Melton *et al.* (1984). The RNA polymerase from bacteriophage SP6 was used to transcribe DNA templates containing an SP6 bacteriophage promoter. Since then, promoter/polymerase systems have been developed for the T7 and T3 bacteriophages as well. These *in vitro* systems require a bacteriophage RNA polymerase, a DNA template encoding a phage promoter upstream of the sequence to be transcribed, an appropriate buffer and ribonucleotides. Each of these components must be ultrapure and free of ribonucleases to prevent degradation of the RNA product once it is transcribed. Conditions have been optimized which generate 100 to 150 µg of RNA from 1 µg of template (MEGAscript US patent #5,256,555). Although *in vitro* transcription is currently the best method of synthesizing long RNA sequences, it is expensive for very large scale production in terms of gram quantities of product due to the large quantities of ultrapure enzymes, nucleotides and buffers needed. Yet, such large quantities of RNA are needed for example in vaccination or gene therapy where transient gene expression is desired.

RNA bacteriophage capsids have been assembled *in vitro* to act as a drug delivery system, called Synthetic Virions (Stockley, 1994). The Operator RNA is synthesized chemically and then conjugated to therapeutic oligonucleotides (antisense DNA/RNA or ribozymes) or cytotoxic agents (ricin A chain). The conjugated Operator is then mixed with Coat Protein *in vitro* to trigger specific encapsidation of the non-phage molecules. The Synthetic Virions are conjugated to ligands which promote uptake in cells by

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receptor mediated endocytosis. Once inside the cell, the Synthetic Virions disassemble and release the therapeutic molecule. The disassembly of the Synthetic Virion is facilitated by the low pH of the endosomal compartments.

RNA has been used to transfect cells *in vitro* and *in vivo* to produce transient expression of the encoded protein. One of the applications for RNA transfection is cancer vaccination (Conry, 1995). RNA has the advantage that expression is transient due to its lability and it is not able to integrate into the host's genome. The use of DNA for nucleic acid vaccination with oncogenes could possibly induce neoplasms. The DNA could integrate into the host genome, leading to a malignant transformation. DNA encoding an oncogene may replicate within cells over periods of months leading to the expression of the oncogene product over the same time period. It has been demonstrated that prolonged expression of some oncogenes in cells may result in their transformation. The current methods for delivering RNA into cells is either injecting naked RNA, cells from tissue culture mixed with naked RNA, or RNA complexed with cationic liposomes into the tissue of an animal (Lu, 1994; Dwarki, 1993). A problem with these delivery systems is the susceptibility of the RNA to ribonucleases in the tissue culture medium or in the extracellular fluids of the host. Transfection efficiency diminishes if the mRNA is degraded before it can reach its target. Transfection efficiency would be improved by increasing the half-life of the RNA prior to its entry into the cell.

SUMMARY OF THE INVENTION

The present invention contemplates various aspects and uses of nuclease resistant nucleic acids. The invention contemplates various methods of making such nuclease resistant nucleic acids. The invention contemplates the use of viral-like systems to produce large amounts of nucleic acid. In preferred embodiments, this nucleic acid is ribonuclease resistant. The invention contemplates the use of nuclease resistant nucleic acids, particularly ribonuclease resistant nucleic acids, in various diagnostic assays.

A primary aspect of the invention is the preparation and use of nuclease resistant nucleic acid standards. Internal standards play an important role in confirming test results. They also provide a means for quantification. The detection and quantification of specific RNAs in samples has become prevalent with the advent of RT-PCR™. The

internal standard for RT-PCR™ studies should be an RNA molecule, as it controls for both the reverse transcription and PCR™ amplification steps. This is problematic, as RNA is particularly susceptible to RNase degradation. Altered test results could be produced by partial or complete degradation of an RNA standard either during storage or after
5 introduction to a sample. The likelihood of at least partial RNA degradation is quite high, given that many of the RNA detection schemes are designed to detect viral RNAs in serum samples, where relatively high quantities of various RNases are located. The ideal internal standard for RNA diagnostic assays is a molecule that is functionally equivalent to RNA in the assay format, but resistant to degradation by nucleases. Three general
10 methods can be imagined for protecting RNA from enzyme-mediated degradation in an environment in which RNases are active: (1) microencapsulating the RNA inside an impenetrable structure, (2) non-covalently binding the RNA with molecules that deny access of nucleases to the standard, and (3) chemically altering the structure of the RNA in such a way that it is no longer a substrate for nucleases while still being functionally
15 equivalent to RNA in the assay format. A more detailed description, examples, and enablements for each are provided below.

The nucleic acids in the standards of the invention can be used in quantifying assays. These standards may be used for a variety of purposes such as quantitative RNA standards (to determine the absolute copy number of a specific RNA sequence),
20 specifically to quantify the number of RNA viruses such as HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, enterovirus, dengue fever virus, or rabies, in plasma, serum, or spinal fluid. They may also be used to quantify the expression of specific mRNA in cells or tissue by an RT-PCR™ assay. The standards may be internal or external. An internal standard is mixed with the sample at a known concentration such that the sample and the
25 standard are processed and assayed as one. Thus, differences in the efficiency of the assay from sample to sample are normalized using the signal generated by the internal standard. An external standard is processed and assayed at a known concentration in parallel with the sample but it is processed separately from the sample. Several different concentrations of the external standard may be processed simultaneously to produce a
30 standard curve which may then be used to determine the value of the unknown sample. Internal and external standards may both be used for quantification but internal standards

are generally regarded as more accurate. The standards may be used as qualitative standards acting as positive controls in diagnostics, for example, bacterial, fungal, or parasitic diseases which are diagnostics RNA based or in RT-PCRTM assays to indicate that all of the reagents are functioning properly. These standards may be used to measure the integrity of an RNA isolation procedure by measuring the amount of degradation observed in the protected RNA after it has been subjected to the isolation procedure followed by Northern blotting. They may be used as environmental tracers to follow the flow of groundwater or to label the waste of individual companies with a unique nucleic acid sequence which can be traced back to the offending company.

The present invention is particularly useful for viral quantification. There are many new nucleic acid based assays in the process of being developed and/or marketed, *i.e.*, Roche Diagnostic Systems, AmplicorTM HIV MonitorTM and AmplicorTM HCV Monitor tests; Organon Teknika, NASBA HIV kit; GenProbe, Transcription Mediated Amplification HIV kit; and Chiron Corp., branched DNA (bDNA) signal amplification assay for HIV and HCV. These assays detect pathogenic human viruses such as HIV and HCV in human plasma or serum. These assays are highly sensitive, detecting even less than 300 virions per 1.0 ml of plasma. In their current format, several of these nucleic acid based assays use naked RNA for their quantitative standards. Unfortunately, these naked RNA standards are very susceptible to ribonuclease degradation and thus the results of the assay may be compromised.

One primary embodiment of the present invention relates to nucleic acid standards comprising nuclease resistant recombinant nucleic acid segments comprising a sequence coding a standard nucleic acid. In some preferred embodiments, the nucleic acid standard is an RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA. As used herein the terms "standard nucleic acid" and "standard RNA" refer respectively to nucleic acids and RNAs that are suitable for use as a standard in the particular assay to be employed. The present invention contemplates a ribonuclease resistant recombinant RNA which is highly suitable as an RNA standard for quantifying RNA viruses, although it need not be recombinant and may be used as an RNA standard for RNA isolated from any source, such as cells from tissue cultures. In particular, the structure of an RNA bacteriophage may be modified to package a

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recombinant RNA (reRNA) molecule. The reRNA sequence serves as an RNA standard for the quantification of a particular RNA sequence/target.

5 In regard to the invention, the terms "nuclease resistant" and "ribonuclease resistant" mean that a nucleic acid exhibits some degree of increased resistance to nuclease over a naked, unmodified nucleic acid of the same sequence.

There are a variety of methods that may be employed to render a nucleic acid segment nuclease resistant. The nucleic acid segment may be chemically modified, coated with a nuclease resistant coating, or caged in a nuclease resistant structure. For example, the RNA standard can be a chemically modified RNA that is resistant to ribonuclease. 10 Another way in which to render a recombinant RNA segment ribonuclease resistant is to coat it with a ribonuclease resistant coating. Such a coating can be anything that binds in a sequence dependent or independent manner to the RNA and renders the RNA ribonuclease resistant. In some cases, the RNA standard is a recombinant RNA that is caged in a ribonuclease resistant structure. Methods of caging RNA involve the partial encapsidation of the RNA in viral proteins, partial lipid encapsulation of the RNA, 15 partially trapping the RNA in polymer matrices, etc.

In some preferred embodiments of the invention, the ribonuclease resistant structure is comprised of a viral Coat protein that partially encapsidates the RNA standard. The RNA is transcribed *in vivo* in a bacterial host and then encapsidated by 20 bacteriophage proteins. This "caging" of the RNA results in RNA which is protected from ribonuclease (Armored RNA™). Although the nucleic acid or RNA may be completely or substantially caged in the nuclease resistant structure, partially caged nucleic acids and RNAs are also within the scope of the present invention as long as the partial caging renders the nucleic acid or RNA nuclease or ribonuclease resistant. Thus, 25 when used herein the terms "encapsidation," "encapsulation," "trapped," etc. encompass structures wherein the encapsidation, encapsulation, trapping etc. is partial as well as substantial or substantially complete so long as the resultant structure is nuclease or ribonuclease resistant as those terms are used herein.

In a specific preferred embodiment, the invention relates to a ribonuclease 30 resistant recombinant RNA ("reRNA") standard. These Armored RNA™ (AR) standards are ribonuclease resistant due to the encapsidation of the reRNA by bacteriophage proteins. The intact RNA is easily extracted from the Armored RNA™ standard particles

by common RNA extraction methods such as the guanidinium and phenol method (Chomczynski, 1987). The non-bacteriophage RNA may be used in many applications: as an RNA standard for quantification, as RNA size standards, and for transient gene expression *in vitro* and *in vivo*.

5 The Armored RNATM can be calibrated to serve as RNA standards in quantitative assays to determine the absolute number of RNA viruses within a plasma sample. The Armored RNATM can be subjected to extreme ribonuclease treatment without any degradation of the RNA standard. Armored RNATM is very durable and can be stored for an indefinite time at 4°C, or even room temperature, in the presence of ribonucleases.

10 There is no known RNA standard with these qualities. Armored RNATM differs in several features from prior art virus-like particles such as those of Pickett and Peabody. The bacteriophage sequence of the reRNA of the Pickett and Peabody particles consisted only of the Operator sequence (or *pac* site) which is required for Coat Protein recognition of the RNA to initiate packaging. The Armored RNATM contains about 1.7 kb of

15 bacteriophage RNA sequence encoding the Maturase, the Coat Protein and the *pac* site. The inclusion of the long stretch of bacteriophage sequence within the packaged reRNA may contribute substantially to forming a macromolecular structure most similar to the wild-type MS2 structure. Further, there may be other, as of yet uncharacterized, sequences within the bacteriophage RNA that recognizes Coat Protein and Maturase that

20 contribute to assembling the bacteriophage particle into a structure that protects the packaged RNA. Non-bacteriophage RNAs can be packaged by Coat Protein alone as demonstrated by Pickett and Peabody but these non-bacteriophage RNA sequences are not entirely ribonuclease resistant. Besides maximizing the possibility of assuming the correct bacteriophage structure, the inclusion of the extra bacteriophage sequence in the

25 Armored RNATM, as opposed to the Pickett and Peabody virus like particles, also increases the specificity of the RNA to be packaged by the bacteriophage proteins. The Pickett and Peabody virus like particles contained mainly the host *E. coli* pre-rRNA over the target RNA unless the ratio of the Coat Protein to reRNA was decreased.

30 A preferred strategy for synthesizing the Armored RNATM is one that has been optimized by producing a self-regulating feedback mechanism to maintain the optimal ratio of Coat Protein to reRNA for assembly. The Coat Protein is encoded in the reRNA and the reRNA is only available for translation in its unassembled form. Thus, when the

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appropriate concentration of Coat Protein has been translated from the reRNA, it begins to package the reRNA. More Coat Protein cannot be translated until more reRNA is transcribed from the recombinant plasmid. The Pickett and Peabody strategy lacked a mechanism for maintaining a constant ratio between these two molecules. Pickett and Peabody used a *trans* mechanism for packaging the Operator-*lacZ* RNA. The Coat Protein RNA was transcribed from a different plasmid and therefore, the Coat Protein was being translated from a different RNA than it was to package. Since there is no Operator on the Coat Protein RNA, the Coat Protein RNA is continually being transcribed and the Coat Protein is continually being translated. After induction, there is no regulation of the synthesis of the Coat Protein. Similarly, there is no control of the transcription of the Operator-*lacZ* RNA. Thus the transcription of both RNAs is constitutive and translation of the Coat Protein is constitutive. In contrast, in some embodiments, the Armored RNATM method is a *cis* method where the Coat Protein is being translated from the same RNA that is to be packaged. The production of the Coat Protein is regulated at the level of translation because once the concentration of Coat Protein is high enough, it encapsidates the RNA from which it is being translated and thus prevents any further Coat Protein from being translated from that RNA. By this autoregulatory method, the levels of Coat Protein cannot become so high that RNA is encapsidated in a non-specific fashion

Armored RNATM may be produced using minimal bacteriophage sequence that encodes the binding sites for Maturase and Coat Protein (or even less) while providing the Maturase and Coat Protein in *trans*. The maximal size of RNA that can be encapsidated and remain ribonuclease resistant remains to be defined. However, the wild type MS2 bacteriophage contains an RNA genome of ~3.6 kb. Since the structure of these bacteriophage is icosahedral, it is likely that the maximal size will be ~4 kb. Thus, the potential to replace the sequences encoding the Maturase and the Coat Protein with a foreign sequence relevant to the user, may be advantageous. One skilled in the art can readily perform a systematic set of studies to determine the minimal amount of bacteriophage sequence necessary to produce Armored RNATM. One advantage of Armored RNATM in these applications is that they are non-replicative and therefore, aberrantly high signals would not be detected due to viral replication.

The stability of Armored RNA™ indicates that the packaged RNA may withstand extreme environmental conditions. This property may be useful in using Armored RNA™ as molecular markers to trace the origin of pollutants. For instance, the Armored RNA™ could be spiked into the waste containers of different companies. The Armored RNA™ for each company would contain a unique nucleotide sequence which would identify that company. In the event of a spill, a sample would be taken, RNA would be isolated and RT-PCR™ performed to determine the unique sequence of the Armored RNA™ and identify the company responsible for the spill. In a related application, the Armored RNA™ could be used by environmentalists to trace the flow of groundwaters.

There are many possible methods of creating genes that, when expressed *in vivo*, will result in Armored RNA™ compositions in which RNA is protected against ribonuclease in a viral Coat Protein synthesized *in vivo*.

In order to understand some aspects of the invention, it is necessary to understand the components of a bacteriophage, for example, the MS2 bacteriophage. The RNA genome is ~3.6 kb and encodes 4 different proteins: the Maturase, the Coat Protein, the Lysis Protein and the Replicase. The Coat Protein composes most of the mass of the MS2 bacteriophage particle. It is a small protein of ~14 kD in size but there are 180 molecules of this protein which encapsidate each molecule of the bacteriophage RNA genome. In total, the Coat Protein molecules provide ~2,500 kD of the total bacteriophage mass of ~3,500 kD. There is one molecule of Maturase protein per bacteriophage particle which is ~44 kD in size. The Maturase serves to protect the RNA genome from ribonuclease degradation and it is the receptor for the F pilus for *E. coli* infection. The Lysis Protein and the Replicase are not a component of the bacteriophage molecule. The Lysis Protein is involved in lysing the *E. coli* cell to release the bacteriophage particles. The Replicase protein and 3 other *E. coli* host proteins compose a protein complex which is responsible for replicating the RNA genome and synthesizing a large number of copies for packaging.

In this application, *cis* refers to a protein binding to the same RNA transcript species from which it was translated. *Trans* refers to a protein binding to an RNA transcript species other than the RNA transcript species from which it was translated.

The simplest composition may be Coat Protein and a RNA encoding a non-bacteriophage sequence either with or without encoding one or more Operator sequences.

Another composition may comprise Coat Protein and a reRNA encoding one or more Operators and a non-bacteriophage sequence.

Another composition may be Coat Protein and Maturase and a reRNA encoding one or more Operator sequences, one or both Maturase Binding sites and non-phage sequence. The Coat Protein and the Maturase are provided in *trans*.

Another composition may be Coat Protein and a reRNA encoding Coat Protein, one or more Operator sequences and non-phage sequence. Including more than one Operator may serve to endow extra protection against ribonucleases and increase the specificity of the Coat Protein for the reRNA over host RNA.

Another composition may be Coat Protein and Maturase and a reRNA encoding Coat Protein, Operator sequence, Maturase Binding site and non-phage sequence. The Maturase protein is provided in *trans*.

Another composition may be Coat Protein and Maturase and a reRNA encoding Coat Protein, Maturase (which includes a Maturase binding sites), one or more Operator sequences and non-phage sequence

Another composition may be Coat Protein and Maturase and a reRNA encoding Coat Protein, Maturase (which includes a Maturase binding sites), one or more Operator sequences, the Maturase Binding Site located at the 3' end of the MS2 genome and non-phage sequence.

Another composition may be Coat Protein and Maturase and a reRNA encoding Coat Protein, Maturase, one or more Operator sequences, most of the C-terminal coding region of the Replicase (so that none of the protein is synthesized *in vivo*). Further, this composition may comprise a sequence coding the entire active Replicase such that the Replicase will function.

Another composition may be Coat Protein and Maturase and a reRNA encoding Coat Protein, Maturase, one or more Operator sequences, most of the C-terminal coding region of the Replicase (so that none of the protein is synthesized *in vivo*) and the Maturase Binding Site located at the 3' end of the MS2 genome and non-phage sequence.

In some embodiments, there will be no need to have an Operator sequence to package RNA. The co-expression of Coat Protein and RNA can lead to operator-less

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RNA being packaged and protected in a manner that may be less specific than produced when an Operator sequence is present.

Other compositions may comprise any of the above, in conjunction with the Lysis Protein. The Lysis Protein may be provided in either.

5 In each of these compositions, the non-phage RNA may be positioned at a variety different regions of the reRNA. For example, in the first composition, the reRNA may encode 2 Operators and non-phage RNA. Both Operators may be located 5' or 3' of the non-phage RNA or there may be one Operator at each end of the non-phage RNA. If there is only a single Operator, it may be preferable to position it at the 3' end of the full-length transcript so that only full-length transcripts are packaged. Including a Maturase Binding Site near the 3' end may have a similar advantage towards packaging full length RNA. In the wild-type phage genome, the Maturase Binding Sites are located within the Maturase coding sequence and at the 3' end of the genome. In the compositions where the Coat Protein is provided in *trans*, it is preferable that there is no Operator sequence encoded on the same RNA transcript as the Coat Protein or the Coat Protein may bind both the Coat Protein RNA transcript and the non-phage-Operator RNA, producing a mixed population of capsids.

15 Using more than one Operator sequence per RNA molecule may be expected to increase the specificity of the Coat Protein for the target RNA and decrease the possibility of packaging host RNA *in vivo* as in the Pickett and Peabody studies. *In vitro* studies investigating the binding kinetics of Coat Protein with the Operator sequence demonstrated that Coat Protein bound in a cooperative manner to an RNA molecule encoding two Operator sequences and that the Coat Protein bound to the RNA with two Operators at a much lower concentration than an RNA with a single Operator (Witherell, 1990). Increasing the specificity of binding may also be accomplished using a mutant Operator sequence with a higher affinity for Coat Protein than the wild type sequence (Witherell, 1990).

20 Major aspects of the invention may be summarized as follows. One primary embodiment of the present invention relates to nucleic acid standards comprising nuclease resistant nucleic acid segments comprising sequences coding a standard nucleic acid. In

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some preferred embodiments, the nucleic acid standard is an RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA.

There are a variety of forms ribonuclease resistant RNA standards that can be employed. The RNA can be chemically modified RNA that is resistant to ribonuclease. A chemically modified RNA may be comprised of chemically modified nucleotides. These nucleotides are modified so that ribonucleases cannot act on the RNA. The chemically modified RNA is prepared by chemical modification of an RNA or a previously transcribed RNA transcript. Alternatively, the chemically modified RNA may be transcribed or synthesized from nucleotides that have already been chemically modified.

An RNA standard may also comprise an RNA that is bound non-covalently, or coated with, a ribonuclease resistant coating. Such binding, which may be sequence dependent or independent, renders the RNA ribonuclease resistant. In some embodiments, the bound molecule is comprised of a protein. Examples of such binding proteins are MS2/R17 coat protein, HIV-1 nucleocapsid protein, gp32, the regA protein of T4, or the gp32 of bacteriophage T4. In other cases, the non-covalently bound molecule is comprised of a small molecule. For example the polyamines, spermine and/or spermidine. The ribonuclease-resistant coating may also be comprised of a nucleic acid. In some preferred embodiments, the nucleic acid hybridizes to the recombinant RNA, blocks nucleases, and can serve as a primer for reverse transcriptase. In other cases, poly-L-lysine and cationic detergents such as CTAB may be used to coat and protect RNA.

In other embodiments of the invention, the ribonuclease resistant RNA segment is a caged ribonuclease resistant structure, that is, the RNA segment is partially encapsulated in a ribonuclease resistant structure. For example, the ribonuclease resistant structure may be comprised of lipids. In some cases, a lipid ribonuclease resistant structure will comprise a liposome. In other embodiments, the ribonuclease resistant structure is a synthetic microcapsule, such as a polymer matrix. Some examples of useful polymer matrices comprise agarose or acrylamide.

In some preferred embodiments, the invention contemplates a nucleic acid standard comprising a ribonuclease resistant structure comprising a standard nucleic acid segment encapsidated in viral Coat Protein. A preferred embodiment of the invention contemplates an RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA. Encapsidation of a RNA segment in a

viral Coat Protein can render it resistant to ribonuclease, hence the term Armored RNA™.

The viral Coat Protein may be any native or modified viral Coat Protein, but, in many preferred embodiments, the viral Coat Protein is a bacteriophage viral Coat Protein. Such bacteriophage viral Coat Proteins may be of an *E. coli* bacteriophage of genetic subclass A or B; in some preferred embodiments, the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage of genetic subclass A. A bacteriophage viral Coat Protein can be of an *E. coli* bacteriophage in serological group I, II, II, or IV, with some preferred embodiments employing a bacteriophage viral Coat Protein from *E. coli* bacteriophage of serological group I. In certain specifically preferred embodiments, the bacteriophage viral Coat Protein is of an MS2/R17 bacteriophage. The bacteriophage viral Coat Protein may also be of a *Pseudomonas aeruginosa* RNA bacteriophage, for example, the *Pseudomonas aeruginosa* PRR1 or PP7 bacteriophage. The bacteriophage viral Coat Protein may further be of a filamentous bacteriophage, and, because such bacteriophage can comprise a longer RNA segment than many other bacteriophage, this is an embodiment of particular interest. It is also contemplated that the bacteriophage of the archae bacteria will be useful in the invention (Ackerman, 1992). Of course, the viral Coat Protein need not be from a bacteriophage, and the invention contemplates viral Coat Proteins from plant or animal virus, for example, tobacco mosaic virus (Hwang 1994a; Hwang, 1994b; Wilson, 1995), the alphaviruses (Frolov, 1996), HBV, feline immunodeficiency virus, and Rous sarcoma virus will all be useful. The viral Coat Protein may be a native or a modified viral Coat Protein. Modified viral Coat Proteins may be used to obtain certain desirable characteristics, such as greater or lesser viral coat resistance. Modified viral Coat Proteins may be made by any of a number of methods known to those of skill in the art, including PCR™-based and other forms of site-directed mutagenesis.

In certain preferred embodiments, the ribonuclease resistant RNA segment is bound to a viral Maturase protein. For example, the RNA standard may comprise a viral Maturase protein bound to a viral Maturase binding site on a recombinant RNA segment. The viral Maturase protein and/or viral Maturase protein binding site may be native or modified. Modifications in the base sequence of the Maturase binding site and in the

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amino acid sequence of the Maturase may be made by any of a number of methods known to those of skill. A viral Maturase binding site is found in the RNA sequence that encodes a native Maturase. Therefore, the RNA sequence may contain within itself an RNA coding for the Maturase. Further, since Maturase binding is purported to have some effect on the stability of RNA segments, it is contemplated that multiple Maturase binding sites and/or Maturase coding sequences may be included in the RNA segment.

The RNA segment which codes for a standard ribonucleic acid may also comprise a sequence coding a Replicase protein, and the Replicase protein may or may not be expressed or expressible from that sequence. In certain preferred embodiments, the sequence coding the Replicase protein codes a modified Replicase protein that is not active.

The RNA segment will typically comprise an Operator coding sequence, and, in many preferred embodiments, a viral Maturase protein binding site which may be included in a viral Maturase protein coding sequence. The RNA segment may further comprise a viral Coat Protein coding sequence of the type discussed above.

There are many embodiments of the RNA segment comprising a sequence coding a standard RNA, a few examples of which are given below. In some very basic embodiments, the RNA segment comprises an Operator sequence and a viral Coat Protein sequence. In other basic embodiments, the RNA segment comprises an Operator sequence, a viral Coat Protein sequence, and a non-bacteriophage sequence. In other embodiments, the RNA segment comprises at least two Operator sequences and a non-bacteriophage sequence. The RNA segment may comprise an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence. Further, in some preferred embodiments, the RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence. The RNA segment may comprise an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence. Alternatively, the RNA segment may comprise an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein, a non-bacteriophage sequence, and a sequence coding a Replicase protein. The

RNA may comprise all or part of the recombinant RNA segment coded for in the sequence of pAR-1 or pAR-2.

In some preferred embodiments, the RNA segment comprises a bacteriophage sequence from an RNA bacteriophage and a non-bacteriophage sequence. The non-bacteriophage sequence may be inserted into a multiple cloning site. The non-bacteriophage sequence may be a viral, bacterial, fungal, animal, plant, or other sequence, although, in certain preferred embodiments it is a viral sequence. Multiple Operators may be on either terminus of the non-bacteriophage sequence, or may flank the sequence. Multiple Operator sequences may be useful for packaging larger non-bacteriophage sequences.

The non-bacteriophage sequence is often a sequence adapted for use as a standard in detection and/or quantification of an RNA by, for example, PCRTM-based procedures. In specific embodiments, the non-bacteriophage sequence is a sequence adapted for use in detection and/or quantification of an RNA of diagnostic value. For example, the non-bacteriophage sequence can be a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen. In some particularly interesting embodiments, the non-bacteriophage sequence is adapted for use in the detection of such viral diseases as HIV-1, HIV-2, HCV, HTLV-1, or HTLV-2. Adaptation of the non-bacteriophage sequence can be accomplished in any manner that will render the sequence suitable for detection and/or quantification of the tested RNA. In some embodiments, the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of an RNA of interest by modifying the native RNA sequence to be detected or monitored so that it is distinguishable from the native sequence. For example, detection and/or quantification of HIV-1 can be accomplished with a non-bacteriophage sequence comprising a modified HIV-1 sequence. The RNA standard may comprise a non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of a blood-borne pathogen, such as a plasmodium, trypanosome, *Francisella tularensis*, or *Wucheria bancrofti*.

The bacteriophage sequence of the RNA standard may be a sequence from any *E. coli* bacteriophage of any genetic subclass, for example, subclass A. Further the

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bacteriophage sequence may be a sequence from an *E. coli* bacteriophage in serological group I, II, III, or IV. In certain embodiments, the bacteriophage sequence is a sequence from an MS2/R17 bacteriophage. Of course, the bacteriophage sequence can also be a sequence from a *Pseudomonas aeruginosa* RNA bacteriophage, such as the PRR1 or PP7 bacteriophage, or a filamentous bacteriophage.

Other embodiments of the invention contemplate a RNA segment comprising various of the sequences discussed above. The RNA standard segment may be encapsidated in viral Coat Protein, or free from viral Coat Protein. For example, a recombinant RNA may be free of viral Coat Protein during the RNA standard production process or during an assay after isolation of the recombinant RNA segment from the viral Coat Protein. The RNA may be of any of the various forms discussed above, and may comprise Operator site(s), Maturase binding site(s), Coat Protein coding sequence(s), Maturase coding sequence(s), non-bacteriophage sequence(s), restriction enzyme sequence(s), active or non-active Replicase coding sequence(s), active or non-active Lysis Protein coding sequence(s) and/or other sequences.

The invention also contemplates DNA vectors adapted for use in the synthesis of a RNA standard comprising recombinant RNA segment encapsidated in viral Coat Protein. Such vectors are transfected into cells, for example *E. coli*, and function to cause the cells to produce RNA encapsidated in viral coat protein. A basic vector may comprise a sequence coding an Operator sequence and a viral Coat Protein sequence. Alternatively, the vector may comprise a sequence coding two Operator sequences and a non-bacteriophage sequence. In some embodiments, the vector may comprise a sequence encoding an Operator sequence, a sequence coding a viral Maturase binding site, and a multiple cloning site. The multiple cloning site may be either downstream or upstream of a sequence encoding a viral Maturase binding site. The vector may further comprise a sequence coding a viral Maturase protein and/or a Maturase binding site. The sequence coding the viral Maturase binding site may be comprised within the sequence coding the viral Maturase protein. Certain preferred embodiments comprise a sequence coding a viral Coat Protein gene, an Operator sequence, and a multiple cloning site. A DNA sequence coding a non-bacteriophage sequence may be inserted into the multiple cloning site of such a DNA vector, and the non-bacteriophage sequence may be any of the sequences discussed above.

The invention contemplates collection tubes containing a nucleic acid standard comprising recombinant nucleic acid encapsidated in viral Coat Protein. Such collection tubes may be adapted for use in collection of a body fluid such as blood, urine, or cerebrospinal fluid. For example, the collection tube may be a vacuum tube for the drawing of blood. Such collection tubes can streamline a diagnostic procedure by providing a nucleic acid standard in a body fluid sample at the time of drawing of the fluid and eliminating the need to add the standard as a part of the assay procedure.

The present invention contemplates methods for assaying for the presence of a tested nucleic acid in a nucleic acid sample using the nucleic acid standards described above. The "nucleic acid sample" may also be described herein as a nucleic acid composition. A nucleic acid composition, as used herein, is taken to mean any composition, usually a liquid composition that contains one or more nucleic acid molecules or polymers. The composition may also comprise buffers, salts, solvents, or solutes and the like, that are derived from a sample along with the nucleic acid composition, or that have been added to the composition during or after isolation. Such compositions are typically precipitated from an aqueous solution and resuspended. The nucleic acid standards may be internal or external standards. Such methods generally comprise the steps of: (1) obtaining a sample; (2) obtaining a nucleic acid standard comprising a nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid; (3) assaying the sample for the presence of a tested nucleic acid sequence; and (4) employing the nucleic acid segment comprising a sequence coding a standard nucleic acid as a standard in the assay. The methods may further comprise the step of isolating a nucleic acid composition from the sample. It is contemplated that samples may include, but would not be limited to inorganic materials such as a soil sample, any organic material, samples from a plant or animal, and may be tissue samples, or samples of blood or blood components. This method may further comprise isolating the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid from a molecule that renders the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid nuclease resistant to obtain a nucleic acid segment comprising a sequence coding a standard nucleic acid.

Alternatively, this method may further comprise admixing the sample or nucleic acid composition and the nucleic acid standard comprising a nuclease resistant nucleic

acid segment comprising a sequence coding a standard nucleic acid prior to assaying for the presence of the tested nucleic acid sequence. This alternative method may further comprise isolating the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid from a molecule that renders the nucleic acid segment comprising a sequence coding a standard nucleic acid nuclease resistant to obtain a nucleic acid segment comprising a sequence coding a standard nucleic acid. Additionally, the sample and the nucleic acid standard may be admixed prior to isolation of a nucleic acid composition from the sample and isolation of the nucleic acid segment comprising a sequence coding a standard nucleic acid so that isolation of the nucleic acid composition from the sample and the nucleic acid segment comprising a sequence coding a standard nucleic acid is performed in the same isolation procedure. This streamlines the procedure and assures that any tested nucleic acid and the nucleic acid standard are processed in parallel in the same reaction. Such parallel processing eliminates many variables that could compromise the results of the assay.

The assay may be any that would employ a nucleic acid standard, although many preferred embodiments comprise PCRTM analysis. One of the advantages of the nucleic acid standards of the invention is that they allow for quantitative assays, such as quantitative RT-PCRTM. In RT-PCRTM procedures, the nucleic acid segment is typically an RNA comprising a sequence coding a standard RNA. Typically quantitative assays will comprise comparing an amount of tested RNA PCRTM product with an amount of standard RNA PCRTM product. RT-PCRTM analysis will usually comprise: (1) employing a reverse transcription procedure; (2) amplifying a nucleic acid sequence and generating a PCRTM product; and (3) detecting PCRTM product. In certain embodiments, the amplification step involves co-amplification of any tested RNA PCRTM product with standard RNA PCRTM product. Such co-amplification can be achieved *via* the use of a single primer set adapted for amplification of both tested RNA PCRTM product and standard RNA PCRTM product from an RT-PCRTM procedure.

The nucleic acid standards may be of any composition described either explicitly or implicitly above. For example, where ribonucleic acid standards are employed any form of ribonuclease resistant RNA segment comprising a sequence coding a standard

RNA may be employed, including, but not limited to those involving chemical modification, ribonuclease resistant coating, or ribonuclease resistant caging.

The sequence coding the RNA standard may comprise a non-bacteriophage sequence, such as a viral sequence. The non-bacteriophage sequence may generally be a sequence adapted for use as a standard in detection and/or quantification of an RNA. In some preferred embodiments, the assay may be employed to detect and/or quantify viral loads in infection with HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen. Presently more preferred embodiments contemplate the detection and/or quantification of HIV-1, HIV-2, or HCV using an RNA standard comprising a recombinant RNA with a modified HIV-1, HIV-2, or HCV sequence.

One specific method of the invention contemplates assaying for the presence of an RNA of diagnostic value by a method comprising: (1) obtaining a sample to be assayed; (2) obtaining an RNA standard comprising a sequence coding a standard RNA encapsidated in a bacteriophage Coat Protein; (3) admixing the sample with the RNA standard; (4) isolating RNA from the admixture; and (4) assaying for the presence of the RNA of diagnostic value with a RT-PCR™ analysis.

The invention contemplates methods of making a nucleic acid standard comprising a recombinant nucleic acid segment encapsidated in viral Coat Protein comprising: (1) obtaining a vector comprising a nucleic acid sequence coding a recombinant nucleic acid segment comprising a sequence coding an Operator sequence, and a non-bacteriophage sequence; (2) transfecting the vector into a cell; (3) providing a viral Coat Protein; and (4) culturing the cell under conditions allowing for transcription of the recombinant nucleic acid segment and encapsidation of the recombinant nucleic acid segment in viral Coat Protein. The recombinant nucleic acid segment may be RNA or DNA. The nucleic acid standard may be purified from the cells in which it is expressed by any of a number of manners known to those of skill for the separation of viral particles from cells. The cell may be any form of cell, although typically a bacterial cell, such as *E. coli* is employed.

Particularly preferred are methods of making RNA standards comprising a recombinant RNA segment encapsidated in viral Coat Protein, which methods comprise: (1) obtaining a vector comprising a DNA sequence coding a recombinant RNA segment

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comprising a sequence coding an Operator sequence and a non-bacteriophage sequence;
(2) transfecting the vector into a cell; (3) providing a viral Coat Protein; and (4)
culturing the cell under conditions allowing for transcription of the recombinant RNA
segment and encapsidation of the recombinant RNA segment in viral Coat Protein. In
5 many preferred embodiments, the recombinant RNA will comprise a Maturase binding
sequence.

The provision of the viral Coat Protein can be by any number of means. For
example, the protein can be expressed separately from the transcription of the
recombinant RNA segment and added into the culture medium in a concentration such
10 that the recombinant RNA becomes encapsidated once transcribed. However, in most
preferred embodiments, the provision of the Coat Protein comprises: (1) obtaining a
nucleic acid segment coding a viral Coat Protein; (2) transfecting the nucleic acid
segment coding the viral Coat Protein into the cell; and (3) culturing the cell under
conditions allowing for expression of the viral Coat Protein. In this embodiment, the
15 nucleic acid segment coding the viral Coat Protein may be a DNA sequence comprised in
the vector comprising the DNA sequence coding the recombinant RNA segment. The
DNA sequence coding the viral Coat Protein may be located *cis* to the DNA sequence
coding the recombinant RNA segment. Further, the DNA sequence coding the viral Coat
Protein can be located in the DNA sequence coding the recombinant RNA segment.
20 Alternatively, the DNA sequence coding the viral Coat Protein may be located *trans* to
the DNA sequence coding the recombinant RNA segment, although this is not typical of
preferred embodiments.

The method of making an RNA standard may comprise the further step of
providing a viral Maturase protein. The provision of the viral Maturase protein can be by
25 any number of means. For example, the protein can be expressed separately from the
transcription of the recombinant RNA segment and added into the culture medium in a
concentration such that the recombinant RNA becomes encapsidated once transcribed.
However, in most preferred embodiments, the provision of the Maturase protein
comprises: (1) obtaining a nucleic acid segment coding a viral Maturase protein; (2)
30 transfecting the nucleic acid segment coding the viral Maturase protein into the cell; and
(3) culturing the cell under conditions allowing for expression of the viral Maturase
protein. In this case, the nucleic acid segment coding the viral Maturase protein may be a

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DNA sequence comprised in the vector comprising the DNA sequence coding the recombinant RNA segment. The DNA sequence coding the viral Maturase protein may be located *cis* to the DNA sequence coding the recombinant RNA segment. Further, the DNA sequence coding the viral Maturase protein can be located in the DNA sequence coding the recombinant RNA segment. Alternatively, the DNA sequence coding the viral Maturase protein may be located *trans* to the DNA sequence coding the recombinant RNA segment, although this is not typical of preferred embodiments. The recombinant RNA sequence, may, of course, be any of those discussed or suggested explicitly or implicitly above.

A preferred embodiment of the method of making an RNA standard comprising a recombinant RNA segment encapsidated in viral Coat Protein comprises: (1) obtaining a vector comprising a DNA sequence coding a recombinant RNA segment comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence; (2) transfecting the vector into a cell; (3) obtaining a DNA segment coding a viral Coat Protein and transfecting the nucleic acid segment coding the viral Coat Protein into the cell; (4) obtaining a DNA segment coding a viral Maturase protein and transfecting the nucleic acid segment coding the viral Maturase protein into the cell; and (5) culturing the cell under conditions allowing for transcription of the recombinant RNA segment, expression of the viral Coat Protein and the viral Maturase protein, and encapsidation of the recombinant RNA segment in viral Coat Protein. In preferred embodiments of this aspect of the invention, the DNA segment coding the viral Coat Protein is comprised in the vector comprising the DNA sequence coding the recombinant RNA segment. More preferably, the DNA sequence coding the viral Coat Protein is located *cis* to the DNA sequence coding the recombinant RNA segment. In preferred embodiments of this invention, the DNA segment coding the viral Maturase protein is comprised in the vector comprising the DNA sequence coding the recombinant RNA segment and, more preferably, located *cis* to the DNA sequence coding the recombinant RNA segment.

The invention also contemplates methods of making RNA *in vivo* comprising: (1) obtaining a vector comprising a DNA sequence coding a recombinant RNA segment comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence; (2) transfecting the vector into a cell;

(3) obtaining a DNA segment coding a viral Coat Protein and transfecting the nucleic acid segment coding the viral Coat Protein into the cell; (4) obtaining a DNA segment coding a viral Maturase protein and transfecting the nucleic acid segment coding the viral Maturase protein into the cell; and (5) culturing the cell under conditions allowing for transcription of the recombinant RNA segment, expression of the viral Coat Protein and the viral Maturase protein, and encapsidation of the recombinant RNA segment in viral Coat Protein. These methods may further comprise the step of isolating the recombinant RNA segment from the Coat Protein, and this allows for the production of a large amount of desired RNA *in vivo*, *i.e.*, within bacterial cells. The isolated RNA segment may then be treated to obtain an RNA segment comprising the non-bacteriophage sequence. For example ribozyme sequences, RNase H and a complementary DNA oligonucleotide that function to generate sequence specific cuts in the RNA, or other molecular biology tools may be used to excise undesired RNA from the non-bacteriophage sequence, or a portion thereof. The desired RNA segment may then be purified by means known in the art. The DNA vectors, RNA segments, cells, *etc.* employed and obtained in this method of *in vivo* transcription may be any of those described above.

The invention also contemplates methods of encapsidating a RNA segment in viral Coat Protein *in vitro* comprising: (1) obtaining a RNA segment comprising a sequence coding a standard RNA; (2) obtaining viral Coat Protein; and (3) placing the RNA segment comprising a sequence coding a standard RNA and the viral Coat Protein together under conditions causing the RNA segment comprising a sequence coding a standard RNA to become encapsidated in the viral Coat Protein.

The invention further contemplates methods of delivering RNA to cells *in vitro* or *in vivo* comprising: (1) obtaining an Armored RNATM comprising a RNA segment comprising a sequence coding a standard RNA encapsidated in viral coat protein; (2) placing the Armored RNATM culture with a cell; and (3) culturing the cell under conditions that cause the Armored RNATM to be taken into the cell.

In such a method, the Armored RNATM may comprise a bacteriophage protein that has been modified to facilitate delivery of RNA to a cell. For instance, the modified bacteriophage protein is a viral Coat Protein or a Maturase protein.

There are many different single and double stranded DNA bacteriophages which infect *E. coli* and other bacteria. Examples of single stranded DNA bacteriophages include

ϕ X174 and M13. Examples of double stranded DNA bacteriophage include T4, T7, lambda (λ), and phage P2. M13 and λ have been used extensively by molecular biologists and it is rather simple to create recombinants of these bacteriophage. As with the RNA bacteriophage, recombinants of the DNA bacteriophage could be constructed and
5 quantified with specific DNA sequences to act as quantitative standards for particular DNA viruses using nucleic acid based assays. Some of the human DNA viruses are HSV, EBV, CMV, HBV, Parvoviruses, and HHV6. The benefit of these standards is that the DNA standards would be protected against DNases.

RNA synthesized by *in vitro* transcription may be packaged with bacteriophage
10 proteins *in vitro*. This method would be useful towards protecting RNA species of very specific sequences, that is, the reRNA would not need to encode the Coat Protein and Maturase sequences. Only the binding sequences for Coat Protein and/or Maturase would be included within the RNA transcript and Coat Protein and/or Maturase would be provided exogenously. Encapsulation may occur co- or post-transcriptionally. It has
15 been demonstrated that by combining RNA and Coat Protein under the appropriate conditions, the RNA will be encapsidated with Coat Protein to form a phage like particle (LeCuyer, 1995). Capsid formation by Coat Protein is stimulated by Operator sequence or long RNA transcripts (Beckett, 1988). Capsids will form without Operator sequence or any RNA but then the concentration of the Coat Protein must be much higher.
20 Therefore, the Coat Protein may be stored at a concentration which does not lead to capsid formation unless it is added to RNA. Using this strategy, the RNA may or may not require the Operator sequence, depending on the length and concentration of the RNA. This strategy may lend itself to packaging mRNA (RNA having a 5' cap and 3' polyA tail) which may then be used in transfection studies (see below). The Maturase may be
25 required to form structures in which the packaged RNA is protected against ribonucleases.

RNA of different discrete lengths may be produced as Armored RNA™ in large quantities. The sizes could be mixed in equal mass amounts in their Armored RNA™ form. This mix may be heated in a denaturing solution and run on a denaturing
30 formaldehyde agarose gel directly or the RNA may be purified from the mix and then run on a denaturing formaldehyde agarose gel as RNA size standards. Alternatively,

chemically modified RNAs of different lengths may be transcribed which are ribonuclease resistant. These RNAs may be used as size standards in gel electrophoresis.

BRIEF DESCRIPTION OF THE DRAWINGS

5 FIG. 1. Consistency of the signal produced by the AR-2 standard which was stored at 4°C. AR-2 standard (80 QS RNA equivalents) was added to 0.2 ml of HIV positive serum and processed using the Amplicor™ HIV Monitor™ test. Twenty one assays were performed over a 38 day period using the same stock of AR-2 standard stored at 4°C in water.

10 FIG. 2. The effect incubating intact AR-2 standards or naked AR-2 RNA in human serum. A defined amount of RNA either encapsidated as AR-2 (500 RNA equivalents), or as naked RNA isolated from AR-2 (5000 RNA equivalents) were incubated with human serum for increasing periods at room temperature (21°C). As controls, the naked AR-2 RNA and the intact AR-2 standard were both incubated in parallel in Tris-buffer. Percent recovery is calculated as the $OD_{450} \times \text{dilution factor (DF)}$ of the samples at time
15 zero divided by $OD_{450} \times \text{DF}$ at the end of each incubation multiplied by 100.

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

The following examples describe the production and use of ribonuclease resistant RNA, illustrate the degree to which the reRNA can be protected from ribonuclease activity, and applications of the invention.

20

EXAMPLE I

Production of Armored RNA™ and Use of Armored RNA™ to Quantify HIV

1. Construction Of Armored RNA™.

25 The Armored RNA™ standard is a modified version of the RNA bacteriophage MS2 which contains only the Maturase and Coat Protein genes. A full-length cDNA clone of the RNA bacteriophage MS2 (pMS27) contains all the genes necessary to produce wild-type, infectious MS2 bacteriophage (Shaklee, 1990).

A fragment of DNA was synthesized from pMS27 by PCRTM encoding the Maturase and the Coat Protein of MS2 using the primers 5' CCTTTCGGGGTCCTGCTCAACTT 3' (sense primer SEQ ID NO: 1) and 5' GATTAGATCTGAGTTGAACTTCTTTGTTGTCTTC 3' (antisense primer SEQ ID NO: 2). A *Bgl*II restriction sequence was incorporated into the antisense primer to mediate the cloning of this PCRTM product into the expression vector pSE380 (Invitrogen Corporation). The PCRTM product was purified using the GeniePrepTM (Ambion, Inc.) and eluted from the glass fiber pad with 70 µl of water. GeniePrepTM is a DNA isolation kit that purifies plasmid DNA based on the common alkaline lysis procedure (Birnboim, 1983). The PCRTM fragment was digested with *Nco* I and *Bgl* II. Ten µl of 10x React 3 Buffer (BRL; 0.5M Tris[pH 8.0]; 0.1M MgCl₂; 1 M NaCl) 20 µl water and 3 µl of *Nco* I and *Bgl* II were added to the purified PCRTM product and incubated 37°C, 2.5h. An *Nco* I site is a naturally occurring sequence within the MS2 genome located just 5' of the start codon for the Maturase gene. The digested PCRTM product was again purified with GeniePrepTM (Ambion, Inc.) and eluted with 70 µl of water.

The digested PCRTM fragment was ligated into the *Nco* I and *Bgl* II sites of vector pSE380. pSE380 previously digested with *Bgl* II and *Nco* I was combined with the *Bgl* II and *Nco* I digested PCRTM product in a 1x Ligation Buffer (50 mM Tris [pH 7.8]; 10 mM MgCl₂; 10 mM DTT; 1 mM ATP; 0.025 mg/ml acetylated BSA, Ambion, Inc.) with T4 DNA Ligase (Ambion, Inc.). The ligation reaction was incubated at room temperature (~21°C), 4 h. Ligated product was transformed into competent *E. coli* strain DH5α cells and then spread onto Cb₁₀₀-LB plates, 37°C, 16 h. Some of the resulting colonies were picked, grown in Cb₂₅-LB medium, the plasmid DNA isolated using GeniePrepTM (Ambion, Inc.) and then screened for a DNA insert with the same size as the PCRTM product by digesting the DNA with *Nco* I and *Bgl* II. The pSE380 vector contains the *E. coli* RNA Polymerase terminator sequence *rrmBT*₁*T*₂, 3' of the multiple cloning site, positions 709 to 866.

The resulting construct is named pAR-1. SEQ ID NO:3. Both bacteriophage genes are downstream of the strong *trc* promoter which is regulated by the *lacI* protein expressed by pSE380. The Operator sequence (*pac* site) is present in the *Nco* I/*Bgl* II fragment just downstream of the Coat Protein sequence. A truncated version of the Lysis

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gene is also present but the peptide encoded is not an active form of the protein. pSE380 encodes *lac I^s*, the superrepressor, which is inactivated in the presence of IPTG. Thus, transcription of the bacteriophage genes is down-regulated until IPTG is added to the culture medium and the *trc* promoter is activated. Transcription is terminated by the
5 *rrnBT₁T₂* terminator sequence.

The Maturase protein, the Coat Protein and the RNA which encoded these proteins were used for the production of the stable, bacteriophage-like Armored RNA™ particle. The Maturase protein is hypothesized to be an important component of Armored RNA™ that is hypothesized to stabilize the bacteriophage-like particle and endow
10 additional protection for the RNA contained within the bacteriophage like particle. Therefore, many preferred embodiments of the invention will include the Maturase protein. There is a binding site for Maturase protein within the Maturase coding sequence (Shiba, 1981). The Maturase binding site was hypothesized necessary to be included in the reRNA as sequence which contributed to the packaging of the reRNA. The Coat
15 Protein is an important component because it makes up the bulk of the bacteriophage particle. It was hypothesized unnecessary to include the Lysis gene or the Replicase gene because neither gene is involved in packaging. The Replicase is not needed because *E. coli* RNA Polymerase transcribes from plasmid DNA the RNA sense (+) strand encoding Maturase and Coat Protein once transcription is induced. However, as mentioned
20 previously, it may not be necessary to have the genes for the Maturase and Coat Protein in *cis* with the RNA standard. They might be supplied in *trans* from another vector or even incorporated into the *E. coli* chromosome.

Armored RNA™ was produced using the pAR-1 recombinant plasmid. *E. coli* strain DH5α harboring this plasmid were grown overnight, 200 rpm, 37°C in LB medium
25 (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl in water, pH 7.0 - 7.5) with 25 µg/ml carbenicillin (Cb₂₅). 0.2 ml of the overnight culture was used to inoculate 2 ml of fresh Cb₂₅-LB medium and incubated 1.5 h, 37°C, 200 rpm. Expression was induced by adding IPTG to 1 mM and incubating 3 h, 37°C, 200 rpm.

The cells were pelleted and then resuspended in 0.25 ml of 5 mM MgSO₄: 0.1 M
30 NaCl: 50 mM Tris (pH 8.0) (Sonication Buffer). The cells were sonicated (Branson Sonifier 450) with the small sonication probe 50% duty cycle, unit 5 power for 5 pulses of

the probe. The sonicate was iced 1 min and then the sonication step was repeated. The sonicate was centrifuged to pellet the cell debris. 20 µl of supernatant was incubated with 100 units of *E. coli* RNase 1 and 2 units of bovine pancreatic DNase 1, 37°C, 40 min, to eliminate *E. coli* RNA and DNA. After nuclease treatment, 15 µl of supernatant was electrophoresed on an agarose gel in TBE buffer and stained with Ethidium Bromide to assay for Armored RNA™.

The Armored RNA™ had a mobility of a 900-1000 base pair double stranded DNA fragment as compared to two different DNA size standards (lambda DNA digested with *Hind*III and pUC19 plasmid DNA digested with *Sau* 3A) which were also run on the gel with the Armored RNA™. The mobility was very similar to the wild-type MS2 bacteriophage mobility. Treatment of Armored RNA™ with DNase or RNase did not affect their intensity of staining or their electrophoretic mobility. Ribonuclease 1 (100 units; Ambion, Inc.) and DNase 1 (2 units; Ambion, Inc.) were added separately or together to 20 µl of the Armored RNA™ supernatant. The supernatant was incubated at 37°C with these enzymes, 40 minutes and then fractionated on an 0.8% agarose gel in TBE buffer. Although the high molecular weight *E. coli* genomic DNA and the *E. coli* RNA were digested by the appropriate enzymes, there was no change to the signal of the Armored RNA™ except that it became more intense and resolved because presence of the *E. coli* nucleic acids in the supernatant tended to smear the signal of the Armored RNA™. However, the nucleases did degrade the genomic DNA and the host RNA.

Cells which were uninduced synthesized some Armored RNA™ but much less than the uninduced cells. Two cultures of pAR-1 in *E. coli* were grown to mid-log phase Cb₂₅-LB. One culture was then induced with 1 mM IPTG and the other was not. Both cultures were grown for another 3 h, 37°C, 200 rpm and then cultures were assayed for Armored RNA™ production. As assayed by gel electrophoresis and Ethidium Bromide staining, more Armored RNA™ was synthesized in the induced cells than in the uninduced. Cells were also induced for 16 h and this protocol resulted in the production of more Armored RNA™ than the 3 h induction. The ribonuclease resistance of the Armored RNA™ containing the 1.7 kb truncated bacteriophage RNA indicates that the full length 3.6 kb of the MS2 bacteriophage genome is not required for the production of Armored RNA™ particles.

2. Construction of an Armored RNATM HIV standard

pAR-1 served as the backbone for the creation of a quantitative HIV RNA standard compatible with the AmplicorTM HIV MonitorTM kit (Roche Diagnostic Systems). This study shows one example of how Armored RNATM can be employed.

5 The QS RNA is the naked RNA standard in the AmplicorTM HIV MonitorTM test. The QS RNA encodes a conserved sequence of the *gag* gene from HIV but also contains a 26 bp substitution of randomized sequence. This random sequence is used to distinguish the wild type HIV amplicon from the QS amplicon since the HIV RNA and the QS RNA are co-amplified in the Monitor test.

10 RT-PCRTM was applied to the naked QS RNA to produce a DNA fragment encoding the QS sequence and contained *Bgl* II and *Kpn*I restriction sites. The primers used were 5' GATTGGTACCTGCTATGTCAGTTCCCCTTGGTTCTCT 3' (SEQ ID NO: 4) and 5' GATTAGATCTAAGTTGGAGGACATCAAGCAGCCATGCAAAT 3' (SEQ ID NO: 5). These primers correspond to the SK431 and SK462 primers
15 respectively used in the Monitor kit (Mulder, 1994) except that a *Kpn* I sequence was added to the SK431 primer and a *Bgl* II sequence was incorporated into the SK462 primer. The QS PCRTM product was digested with *Bgl* II and *Kpn* I and ligated into the *Bgl* II and *Kpn* I sites in pAR-1 creating the recombinant plasmid, pAR-2 SEQ ID NO:6. DNA sequence of the *Nco* I/*Kpn* I fragment of pAR-2.

20 An *Nco* I/*Kpn* I fragment containing the Maturase, the Coat Protein and the QS RNA sequence was cloned into the *Nco* I/*Kpn* I restriction sites of the expression vector, pSE380 (Invitrogen Corp.) to produce pAR-2, SEQ ID NO:6. The important regions of the insert include: *Nco* I (1>6), *Bgl* II (1713>1718), *Kpn* I (1862>1867), Maturase coding sequence (53>1231), Coat Protein coding sequence (1258>1647), Lysis Protein
25 (1601>1711), the Capture Sequence within the QS sequence (1757>1782), the QS Amplicon region (1720>1861), SK462 primer (1720>1749), SK431 primer (1861>1835), Maturase Binding Site (311>337), and Operator sequence or *pac* site (1667>1687). SEQ ID NO:6.

30 pAR-2 in *E. coli* was induced with 1 mM IPTG in Cb₂₅-LB at mid-log phase for 3 h, 37°C, 200 rpm. The induced cells were pelleted and then sonicated in the Sonication Buffer used for the AR-1 particles. Then cell debris was pelleted by centrifugation. The

sonicate supernatant was incubated with RNase 1 (5 units/ μ l) and DNase 1 (0.1 units/ μ l), 37°C, 30 minutes and then fractionated on an agarose gel and visualized by Ethidium Bromide staining. The AR-2 particles were detected as a fluorescent band migrating at about 900 base pairs as compared to the DNA markers run on the same gel.

5 The AR-2 sonication supernatant containing the AR-2 particles was incubated under the same conditions as above with RNase 1 and DNase 1 except that the incubation was for 16 h. Another sample of AR-2 was incubated 16 h, 37°C but nucleases were not added. These samples were fractionated on an agarose gel and compared to the AR-2 preparation which was stored at 4°C. The AR-2 signal was strongest in the sample which
10 was treated with the nucleases because the nucleases degraded the nucleic acids which were masking the AR-2 signal.

 The AR-2 were also isolated from culture supernatant. pAR-2 transformed *E. coli* were induced with 1 mM IPTG, 16 h in 50 ml of Cb₅₀-LB medium. Inoculation and induction were simultaneous. 0.4 ml lysozyme (50 mg/ml) was added to the culture and
15 incubated 37°C, 200 rpm, 1 h followed by the incubation of the cell culture with 0.4 ml of 1-bromo-3-chloro-propane 37°C, 200 rpm, 10 min. The cell debris was pelleted by centrifugation in an SS34 rotor, 4°C, 9000 rpm, 10 min. The culture supernatant was transferred to fresh tubes and 0.015 ml of 1-bromo-3-chloro-propane was added. The culture supernatant was stored at 4°C. 0.015 ml of the culture supernatant was
20 fractionated on an agarose gel and detected by ethidium bromide staining and UV fluorescence. AR-2 were detected in the supernatant as well as genomic DNA from *E. coli*. However, unlike the AR-2 isolated from *E. coli* cytoplasm, this AR-2 preparation did not contain detectable *E. coli* RNA. A 0.5 ml prep of AR-2 from the spent growth medium was treated with 10 units of DNase I, 37°C, 70 min. As assessed by gel
25 electrophoresis, DNase I completely degraded the host DNA but did not affect the AR-2.

 0.02 ml of the AR-1 and AR-2 particles isolated from *E. coli* cytoplasm by sonication, were treated with 2 units DNase I and 100 units RNase 1, 37°C, 2 h and then 21°C, 16 h to degrade all host *E. coli* RNA and DNA. The AR preps were subjected to single tube RT-PCR™ using primer pairs specific for the QS amplicon, SK431 and
30 SK462 (Mulder, 1994), and specific for the MS2 bacteriophage sequence, MS2-1 and MS2-2. The Armored RNA™ preps were diluted 10, 100 and 1000 fold in PBS. RT-

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PCRTM was performed with 1 µl of each of the Armored RNATM dilutions using SK431/SK462 and MS2-1/MS2-2 primer pairs. Prior to the reverse transcription step, the Armored RNATM preps were incubated at 95°C, 5 min to disrupt the protective protein coat of the Armored RNATM. The RNA was cooled on ice in the presence of the primers and then incubated with 100 units MMLV-RT, 42°C, 1 h. 2.5 µl *Taq* polymerase was added, followed by PCRTM. The PCRTM products were fractionated by gel electrophoresis. Only the AR-2 generated the expected PCRTM product of 142 bp with the SK primers whereas the AR-1 and AR-2 generated a 411 bp product using the MS2 primers. These results were consistent with the AR-2 particles containing the QS RNA target and an MS2 RNA target while the AR-1 only contain the MS2 sequence.

Of course, the above protocol can be modified to produce almost any RNA standard by cloning the DNA sequence (encoding the RNA sequence of interest) into a recombinant plasmid such as pAR-1. SEQ ID NO:3. This plasmid encodes the Maturase, the Coat Protein and the Operator sequence and there are many convenient restriction enzyme sites immediately downstream (3') of the *Bgl* II restriction site of pAR-1. These restriction sites are the same restriction sites originally encoded in the multiple cloning site of pSE380, from nucleotides 396 to 622. Thus, DNA fragments may be synthesized chemically for cloning into pSE380. Alternatively, PCRTM or RT-PCRTM may be used to synthesize long DNA fragments (>100 bp) using primers which introduce restriction sites at both termini of the DNA fragment. The PCRTM DNA fragments may be digested for cloning into pAR-1.

An advantage of using the larger gene fragments is that PCRTM primers to different regions of these genes may be used with a single Armored RNATM standard and it is not necessary to construct a different Armored RNATM standard for each PCRTM primer pair that might be used. If large fragments of HIV RNA sequence (1 to 3 kb) are packaged into Armored RNATM, then the user has the option of using a variety of primer pairs for performing RT-PCRTM. This type of construct may more readily conform to the primer pairs that the researcher is currently using and he would not need to change primer pairs in order to use the Armored RNATM standard. The user is not limited to just one set of primer pairs as when using, for example, the short 142 base pair region used in the HIV monitor assay.

Of course, the HIV sequences may contain modifications from the wild-type sequence which will allow the standard sequence to be distinguished from the wild-type for the purpose of using the Armored RNA™ in competitive PCR™ as quantitative standards. Such modifications include insertions, deletions and restriction enzyme sequences.

It is not known what is the maximum limit of non-bacteriophage RNA can be packaged as an Armored RNA™. However, the full length genome of MS2 is ~3.6 kb and pAR-1 encodes only ~1.7 kb of the MS2 genome. Therefore, it is very likely that as a minimum, at least 2 kb of non-bacteriophage RNA sequence can be encapsidated as Armored RNA™ and possibly more if the Maturase and Coat Protein genes are supplied in *trans*.

3. AR-2 used as a quantitative RNA standard for HIV

In the Amplicor™ HIV Monitor™ test, the naked QS RNA standard is added to the Lysis buffer just prior to using the Lysis buffer for isolating the HIV RNA from plasma or serum. It is in this manner that the QS RNA is added to the plasma sample and that the HIV RNA and the QS RNA are co-purified. After isolation, the RNA is subjected to RT-PCR™ using *Tth* DNA polymerase which functions as a reverse transcriptase and a heat stable DNA polymerase. After RT-PCR™, the PCR™ products are incubated in the wells of an ELISA plate which contain oligonucleotides (capture probes) immobilized to the well bottoms. One set of wells has an oligonucleotide sequence which is complementary to the wild-type sequence of HIV. The other capture probe recognizes the unique sequence in the QS amplicon. Thus, the HIV capture probe hybridizes to the HIV amplicon while the QS capture probe hybridizes to the QS amplicon which result from the RT-PCR™ co-amplification. After hybridization, the amount of amplicon hybridized is detected enzymatically and colorimetrically using horse radish peroxidase. In the standard assay format recommended by the manufacturers, about 60 to 80 copies of the QS RNA standard are added to each sample for analysis, depending on the kit lot number.

To test Armored RNA™ in the Amplicor™ HIV Monitor™ test, the naked QS RNA, and the concentrated AR-1 and the AR-2 were subjected to the Amplicor™ HIV

Monitor™ test. The QS RNA, AR-1 and AR-2 were added to the Lysis buffer, the RNA was isolated and then subjected to the RT-PCR™ procedure. The PCR™ products were detected on assay plates. The QS RNA produced the expected signal, the AR-1 were negative whereas the AR-2 produced a signal so strong that it was off-scale. Once the concentrated AR-2 prep was diluted 30 million fold, the signal became comparable to the QS RNA standard. The AR-2 prep was diluted by 30 million to produce a stock of Armored RNA™ which was the equivalent of 8,000 copies of QS RNA per milliliter. 10 µl of this AR-2 stock produced the same signal in the Amplicor™ HIV Monitor™ assay as the recommended amount of the naked QS RNA standard provided with the kit.

The AR-2 were diluted in water and stored at 4°C over a period of several months. The diluted AR-2 were calibrated to produce a signal similar to the QS RNA in the Amplicor™ assay. Numerous Amplicor™ assays have been performed with the same diluted stock of AR-2. During a 38 day period, there has been no detectable decrease in the signal produced with the AR-2, highlighting the durability of these RNA standards (FIG. 1). This result is remarkable considering that this AR-2 stock was a crude preparation from *E. coli* and contained ribonucleases.

4. Durability of Armored RNA™ compared to naked RNA

One advantage of Armored RNA™ is that rather than adding the Armored RNA™ standard to the lysis solution prior to the lysis procedure, the Armored RNA™ standard may be added to the plasma or serum sample prior to RNA isolation, thus minimizing pipetting error in the assay. Armored RNA™ withstands plasma/serum nucleases very well compared to naked RNA.

10 µl RNA purified from AR-2 (about 5000 QS RNA equivalents), and intact AR-2 (about 500 QS RNA equivalents) were each added to 0.2 ml of normal human serum and incubated, 21°C for 0 sec, 15 sec, 30 sec, 1 min, 5 min, 15 min, 1 h, and 4 h. The incubations were stopped by the addition of 4M guanidine thiocyanate, and 1% Sarkosyl solution and the RNA was extracted from the serum according to the method of Chomczynski (1987). Fifty microliters of a purified RNA preparation was amplified by RT-PCR™ for 26 cycles using the SK431/SK462 primer pair. The PCR™ products were quantified with a solid phase ELISA system (Mulder, 1994).

The AR-2 generated the same signal over the full time course while the signal from the purified RNA from the AR-2 disappeared almost immediately (FIG. 2). Clearly, the RNA in AR-2 was protected against plasma ribonucleases compared to the naked QS RNA.

EXAMPLE II

Use of Armored RNA™ to Quantify HIV

1. Use of Armored RNA™ Standard in the Amplicor™ HIV Monitor™ Assay

To perform the Amplicor™ HIV Monitor™ assay, a quantitative RNA standard of a known quantity is added to the patient's plasma sample and then RNA is isolated from the plasma. RT-PCR™ is performed on the RNA such that the standard and the HIV RNA are co-amplified using a single primer set. Both PCR™ products are measured and then the concentration of the HIV RNA is calculated using the signal obtained from the quantitative standard.

The Armored RNA™ standard containing the QS sequence (AR-2) is used in the Amplicor™ HIV Monitor™ test as follows. A known quantity of AR2 (~10 µl) is added to 0.2 ml of sample plasma. 0.6 ml of Lysis Reagent is added to the plasma sample containing the AR-2. The sample is mixed by vortexing 3-5 seconds and then incubated at room temperature 10 minutes. 0.8 ml of isopropanol is added to the sample tube and the sample is mixed by vortexing 3 to 5 seconds. The sample is centrifuged at maximum speed (~16,000 × g) for 15 minutes. The supernatant is discarded without disrupting the pellet. 1 ml 70% ethanol is added, and the sample is vortexed 3 to 5 seconds followed by centrifugation for 5 minutes at ~16,000 × g.

The pellet is resuspended in 0.4 ml of Specimen Diluent. 50 µl of the extracted sample is added to the MicroAmp tube containing the Master Mix and RT-PCR™ is performed using *Tth* Polymerase.

After amplification, 0.1 ml of Denaturation Solution is added to the amplicons. 0.1 ml of Hybridization Solution is placed into each of well of the Microwell Plate (MWP) used for detection of the amplicons. 25 µl of the denatured amplicon is added to the first well and 1:5 dilutions are performed using 25 µl in the next 4 consecutive wells such that there are dilutions of 1:5, 1:25, 1:125, 1:625 and 1:3125 for the detection of the HIV

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amplicon. Dilutions of 1:5 and 1:25 are made in the appropriate wells for the detection of the QS amplicon. The MWP is incubated for 1 hour at 37°C.

The wells are washed 5 times with Working Wash Solution and then 0.1 ml of AV-HRP is added to each well and incubated 15 minutes, 37°C.

5 The MWP is washed 5 times with the Working Wash Solution. Then, 0.1 ml of the Working Substrate is added to each well and the MWP incubated 10 minutes, in the dark, at room temperature. 0.1 ml of Stop Solution is added to each well and the optical density is measured at 450 nm. The concentration of the HIV in the plasma is calculated based on the signal obtained from the known concentration of the QS standard added to
10 the patient plasma.

Besides using Armored RNA™ as a substitute for the naked QS RNA standard in the HIV Monitor™ assay, Armored RNA™ may also be used as a positive control in the assay. A wild-type sequence of HIV compatible with the HIV Monitor™ test could be packaged in Armored RNA™ such that it behaves as if it were a wild-type HIV. In this
15 embodiment, an Armored RNA™ HIV Positive control is added to normal plasma at a known quantity and is then processed as if it were a patient sample except that the user would expect to obtain a certain pre-determined value in the assay. This standard would be used to demonstrate to the user that the assay was functioning properly.

2. Modified Amplicor™ HIV Monitor™ test procedure well suited to Armored RNA™

20 To increase the sensitivity of detection for HIV in the Amplicor™ assay, a procedure was developed in which the HIV virions are pelleted by high speed centrifugation from the plasma sample. Thus, 1 ml of plasma can now be assayed instead of the conventional 0.2 ml which the assay has used. This procedure should theoretically increase the sensitivity 5 fold, if the centrifugation step pellets 100% of the HIV virion.
25 This protocol is only useful as a qualitative assay for HIV.

In this new procedure, the naked QS RNA would not be an optimal standard. It cannot be added to a plasma sample due the ribonucleases in the plasma which would degrade it and the naked QS RNA would not pellet by centrifugation with the HIV virions. Thus, when the plasma is removed after centrifugation, there is no control for
30 loss of the HIV pellet. Armored RNA™ may be added to the plasma prior to

centrifugation. It should pellet similarly to the HIV. Thus any loss of the pellet would be reflected in an equal loss in signal obtained from the Armored RNA™ standard. Importantly, the use of Armored RNA™ standards can convert the centrifugation protocol from a qualitative assay to a quantitative assay.

5 3. Use of Armored RNA™ in Other Assays

Of course, the invention is not limited to a single, exemplary assay. There are other RNA based assays for HIV including: NASBA which is based on isothermal amplification (van Gemen, 1994; sold by Organon Teknika); the branched DNA assay developed by Chiron; an assay by DiGene in which an antibody recognizes a DNA/RNA
10 duplex; and transcription mediated amplification, a technology similar to NASBA developed by Gen-Probe. For each of these assays, one skilled in the art can construct an Armored RNA™ standard containing appropriate sequence(s) to function appropriately as a quantitative standard.

The HIV NASBA assay uses three different quantitative RNA standards. The
15 sequences of these standards are available in van Gemen (1994). Each of these NASBA standards could be cloned into pAR-1 in the same manner that the QS sequence was cloned into pAR-1 to produce Armored RNA™ standards for the NASBA assay. RT-PCR™ may be used to amplify each of the standards for cloning into pAR-1.

The branched DNA assay by Chiron uses a single stranded DNA as the RNA
20 standard. It encodes the *gag* and *pol* genes of HIV strain SF2. The sequence for this strain of HIV is available from GenBank, Accession number K02007. This HIV standard sequence is ~3 kb and may be too long for its full length to be packaged using pAR-1 as the vector for Armored RNA™ synthesis. It may be necessary to use a different construct which may permit longer sequences to be packaged. One such Armored
25 RNA™ composition would be comprised of Maturase protein, Coat Protein and a reRNA coding Coat Protein, Operator site(s) Maturase Binding Sites and the HIV sequence encoding *gag* and *pol*. The Maturase would be encoded on another plasmid provided in *trans*. In this construct, the deletion of the Maturase Coding sequence from the reRNA may permit for the packaging of more non-bacteriophage sequence.

EXAMPLE III

Use of Armored RNA™ in a HIV Gel-Based Assay

An Armored RNA™ may be used as an external quantitative standard. A fragment of the HIV genome such as that region bounded by the SK431 and SK462 primers or some other region such as sequence encoding the *pol* gene can be cloned into the pAR-1 backbone. This construct can be used to synthesize Armored RNA™ in which the entire length of the cloned HIV fragment is wild-type HIV sequence. Such an assay does not require a unique capture sequence, because each amplicon is detected on an acrylamide or agarose gel either by ethidium bromide staining or by labeling the PCR™ product with ³²P incorporation and autoradiography. A standard curve is generated by introducing known amounts of the HIV sequence in to the RT-PCR™ assay and then quantifying the amount of product generated on a gel. Actual copy numbers for test samples may then be derived using the standard curve. The Armored RNA™ is calibrated and used in RT-PCR™ reactions as if it were actual HIV. The PCR™ fragments may be fractionated either by agarose or acrylamide gel electrophoresis and quantified by ethidium staining or radioactivity. Standard curves may be generated by processing plasma containing different concentrations of the Armored RNA™. The standard curve may then be used to calculate the titers of patient samples by interpolation.

EXAMPLE IV

Use of Armored RNA™ as a Non-Infectious Standard

Armored RNA™ may be used as non-infectious proficiency standard (a certified and well characterized plasma, serum or urine based product designed for the validation of the accuracy of the instrumentation and methods of an assay) or as positive controls. Gene fragments of a standard HIV strain are cloned into the Armored RNA™ recombinant plasmid to produce a family of Armored RNA™-HIV standards. These include the *pol* and *gag* genes, which are well conserved in HIV. The inventors envision AR-*gag*, AR-*pol* and AR-*gag/pol* RNA standards. These Armored RNA™ standards are quantified precisely and then can be used as external quantitative standards for RT-PCR™ or other amplification techniques. The Armored RNA™ may be added directly to plasma

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and used as such, or the AR-HIV RNA may be extracted from the purified Armored RNA™ in a standard salt buffer such as PBS or Tris:NaCl.

EXAMPLE V

An Armored RNA™ Standard Used in an HCV Assay

5 Armored RNA™ technology will be useful in creating RNA standards for viruses that are difficult or hazardous to culture. For example, hepatitis C virus (HCV) cannot be reliably grown in tissue culture, whereas an Armored RNA™ standard for HCV could easily be constructed and produced. HCV isolates can be classified into 6 distinct genotypes based on the nucleotide sequence variation in the core gene region or the NS5
10 region (Simmonds, 1994). Genotype-specific Armored RNA™ standards can be constructed to serve as controls in assays designed to identify specific HCV genotypes and HCV subtypes such as 1a, 1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, and 6a. Genotyping strategies have been based on sequencing, RFLP analysis, PCR™-based assays with type-specific primers, and a line probe assay (van Doorn, 1994). For example, the latter assay involves
15 the differential capture of a 244 basepair product that is generated from primers at positions 56 through 299 of the HCV genome. The amplicon used to identify each of these strains is short enough that it is straightforward to chemically synthesize the DNA fragments to be cloned into pAR-1 and thus circumvent the need to handle infectious material in order to clone these genotype sequences. Protected recombinant RNA from
20 human serum samples could be constructed using primers KY80 (5'GCAGAAAGCGTCTAGCCATGGCGT) (KY80 -SEQ ID NO: 7) and KY78 (5'CTCGCAAGCACCTATCAGGCAGT) (KY78 -SEQ ID NO: 8). By using samples with defined genotypes, genotype-specific recombinant standards could be constructed. Armored RNA™ standards for HCV may be constructed using a strategy similar to the
25 one for the HIV standard. These RNA standards may be used either as positive controls for strain typing or quantitative RNA standards.

For strain typing, the primers KY78 and KY80 may be used to synthesize DNA fragments by RT-PCR™ from different HCV strains. These strain specific DNA fragments may be cloned into pAR-1 such that RNA encoding strain specific HCV is
30 packaged as Armored RNA™. As well, strain specific sequences have also been

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documented for HCV between positions 56 to 299 of the HCV genome. It is possible to chemically synthesize these sequences and clone them directly into pAR-1 for packaging. This method would circumvent the need to handle infectious HCV. Thus, in the differential capture assay, each strain specific PCRTM product of HCV would hybridize to a unique capture probe immobilized to the bottom of a plastic well.

To create a quantitative HCV Armored RNATM standard, the amplicon produced by the KY78 and KY80 primers could be modified by substituting in the same capture sequence used in the QS sequence for HIV or some other 25 to 30 base pair region within this amplicon may be randomized so that it can be differentiated from the wild-type amplicon. The substitution may be performed by one skilled in the art by PCRTM. As in the AmplicorTM HIV assay, the HCV quantitative standard would be co-amplified with the wild-type RNA. The HCV Armored RNATM standard would be added to the sample plasma prior to the RNA isolation step.

EXAMPLE VI

Collection Tubes Containing Pre-Determined Quantity of Armored RNATM

Owing to the durability and stability of Armored RNATM, it may be aliquoted into blood collection vessels or other fluid collection vessels in a pre-determined quantity. The Armored RNATM may then be freeze dried for long term storage at room temperature or left in buffered salt solution and stored at 4°C or even room temperature (~21°C). At the time of use, blood is drawn into the collection tube with the Armored RNATM standard. The blood sample is inverted many times to thoroughly mix the Armored RNATM standard into the sample. The blood may be stored as usual until it is subjected to the quantitative assay. This strategy precludes the need to add an RNA standard to the plasma sample prior to the RNA isolation from plasma. It would control for the partitioning of the viruses between the blood cells and the plasma or serum fractions.

Further, multiple Armored RNATM standards may be included in the collection tube such as an HIV and an HCV standard so that the quantification for one or both pathogens may be performed.

EXAMPLE VII

Use of Armored RNA™ in Veterinary Diagnostics

Domestic animals are often infected with RNA viruses. For cats, retroviruses represent the largest cause of premature death other than automobile accidents. Up to one-third of the cats exposed, are infected with Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) (Essex, 1995), similar to their human counterparts, HIV and human T cell lymphotropic virus-type 1 (HTLV). One skilled in the art could construct Armored RNA™ standards for FIV and FTLV, similar to the AR-2 standard described for HIV. These standards may be used for quantification or as positive controls in FIV and FeLV diagnosis by RT-PCR™.

EXAMPLE VIII

Use of Double Stranded RNA Bacteriophage

Bacteriophage $\phi 6$ is a double stranded RNA bacteriophage in which it is possible to package *in vitro*, non-bacteriophage RNA to produce viable, genetically stable bacteriophage (Qiao, 1995). It has been shown that bacteriophage $\phi 6$ has a sequenced genome of three separate pieces associated with a nucleocapsid. The nucleocapsid comprises five component proteins, with once, P8 forming an outer shell and the four others, P1, P2, P4 and P7, forms an inner particle. The $\phi 6$ nucleocapsule can be assumed *in vitro* (Olkkonen, 1990). It has been demonstrated that the RNA in these bacteriophage are protected from ribonucleases. One skilled in the art may produce recombinants containing a standard RNA sequence so that the recombinant bacteriophage $\phi 6$ may act as quantitative standards for human infectious, double stranded RNA viruses such Rota Virus and Vesicular Stomatitis Virus. It is possible following the teachings of this specification and the art to package with bacteriophage $\phi 6$ whereby the packaged material is not viable/infectious, similar to the Armored RNA™ standards. For example, additional RNA sequences may be added to or substituted into the bacteriophage genome. Other double-stranded RNA bacteriophage can be similarly employed.

A double stranded non-infectious RNA bacteriophage may have applications as a therapeutic. Double stranded RNA is known to stimulate cells to produce important immunomodulators which act as anti-viral agents such as interferon (Stiehm, 1982).

EXAMPLE IX

Quantification of a Cellular mRNA

One skilled in the art can use an Armored RNATM standard for the quantification of an mRNA expressed by a cell. In studies to determine the induction or repression of specific mRNAs over a period of time, an equal number of cells could be harvested at each time point before and after the cells had been exposed to the treatment under investigation. The Armored RNATM standard would be added in a known quantity to each time point prior to the purification of the RNA from the cells. The Armored RNATM standard could then be used to quantify the amount of a target mRNA such as a cytokine, a cell cycle gene or an oncogene. The Armored RNATM standard would be constructed containing the same primer pair binding sites as the target gene. The Armored RNATM standard could be differentiated from the wild type RT-PCRTM product by altering the sequence between the primers pairs using one of the methods discussed above such as incorporating a restriction site or a deletion. One skilled in the art can produce amplicons which have insertions, deletions or substitutions as compared to the wild-type sequence. There are several different methods available for site-directed mutagenesis, each of which involve oligonucleotides which encode the desired sequence of the standard

Standards containing deletions, insertions and restriction sites are often preferred for gel based assays because they are easily differentiated from the wild-type amplicon by fractionation on an agarose or acrylamide gel. For each of these standards, a size difference of about 10% is often used. PCRTM may be used to generate any of these standard sequences. To generate a deletion mutant, an oligonucleotide is synthesized which contains primer sequences as well as other amplicon sequence. The amplicon sequence and the primer sequence flank the sequence in the wild-type sequence which is to be deleted. When this oligonucleotide hybridizes to the wild-type, the sequence to be deleted is looped out and is not incorporated into the new DNA strand during polymerase extension. One skilled in the art may use similar strategy to create insertions and substitutions whereby one of the primers in a PCRTM reaction contains the desired mutation. Two such strategies are discussed in detail in Schneeberger (1996) and Hughes (1996).

EXAMPLE X**Preparation of Armored RNA™ Standards for Commercial Use**

It is important that the Armored RNA™ standard is free of any host DNA or RNA which may affect quantification by RT-PCR™. To produce a homogenous lot of an Armored RNA™ standard, a crude extract would be prepared from the culture supernatant after a 16 h induction of the *E. coli* transformants (see EXAMPLE I). This preparation is contaminated with the genomic DNA from *E. coli*. Contaminating RNA and DNA may be removed by adding RNase and DNase to the crude extract.

Traditionally, MS2 bacteriophage are purified by a CsCl gradient. They band tightly at a concentration of 1.45 g/cc (Pickett, 1993). Armored RNA™ may be purified by using a CsCl gradient procedure similar to the MS2 bacteriophage. After centrifugation, the Armored RNA™ band is pulled and then dialyzed against a salt buffer such 100 mM NaCl: 50 mM Tris (pH 7.5) or PBS. Armored RNA™ may be quantified by obtaining an OD₂₆₀ and an OD₂₈₀ which are used in the art to measure nucleic acid and protein concentrations, respectively. After a stock of Armored RNA™ has been made, it may be calibrated against a naked RNA containing the same amplicon. For example, Armored RNA™ standards may be calibrated against the naked QS RNA standard.

The nuclease treated crude extract of Armored RNA™ may be also purified by gel exclusion chromatography, using a resin such as Sephacryl S-200 (Pharmacia) (Heisenberg, 1966). Due to the large size of the Armored RNA™, they will run in the void volume while other protein and nucleic acid components will be retarded. Armored RNA™ in the void volume may be calibrated as above. It will be advantageous to couple several purification procedures to ensure that the Armored RNA™ is completely homogenous.

Large scale production of Armored RNA™ may be performed as follows. A 100 ml inoculum of *E. coli* harboring the pAR construct is grown to mid-log phase in Cb₂₅-LB medium. This inoculum is used to inoculate 1,000 ml of 1 mM IPTG- Cb₂₅- LB medium. The cells are incubated 16 h, 200 rpm, 37°C. One ml of lysozyme (50 mg per ml) is added to the culture and incubated 37°C, 1 h, 200 rpm. The culture is pelleted by centrifugation. The supernatant contains Armored RNA™ particles and *E. coli* genomic and plasmid DNA. Contaminating RNA and DNA is degraded by adding CaCl₂ to the

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supernatant to 10 mM and the supernatant is then incubated with Micrococcal Nuclease (30 units/ml), 37°C, 1 h, 200 rpm. EDTA is added to 25 mM to chelate the CaCl_2 and stop the nuclease activity. The AR particles are precipitated with 50% Ammonium Sulfate, 4°C, 2 h. The precipitate is pelleted by centrifugation. The pellet is resuspended in 100 mM NaCl: 1 mM EDTA: 10 mM Tris (pH 7.5) (TSE buffer). 0.6g of CsCl is added to every gram of AR2 solution. The CsCl is dissolved and transferred to a heat sealed ultracentrifugation tube for the 50.2 Ti rotor (Beckmann). The CsCl gradient is centrifuged at 45,000 rpm, 20 h, 21°C. The AR particles band about the middle of the centrifuge tube. The CsCl band is pulled with a needle and syringe, about 5 ml. The AR particles are finally passed over an Sephacryl S-200 resin in TSE buffer. The particles elute in the void volume. The number of AR2 particles may be determined using the extinction coefficient of 1 $\text{OD}_{260}=0.125$ mg/ml of MS2 bacteriophage and the molecular weight is 3×10^6 . Based on this procedure, approximately 1×10^{15} AR particles can be purified from one liter of culture.

Electron microscopy may be used to count the Armored RNA™ directly. This method has been used for quantifying HIV (Lu, 1993).

After purification, the Armored RNA™ may be stored at 4°C or at room temperature. A biocide may be added to prevent bacterial or fungal growth in the standards. The Armored RNA™ is diluted to concentrations which are convenient for quantification.

EXAMPLE XI

Use of other RNA bacteriophage for constructing Armored RNA™

There are 3 other genetically distinct groups of RNA coliphage and at least 2 other non-*E. coli* RNA bacteriophage, PP7 and PRR1 (Dhaese, 1979; Dhaese, 1980). These bacteriophage have Coat Proteins which are homologous in protein sequence to the MS2 Coat Protein (Golmohammadi, 1993). As with the MS2 phage, the cDNA of the Maturase and the Coat Protein of any of these bacteriophage could be cloned into an expression vector such as pSE380. These recombinant plasmids may act as vectors into which non-phage DNA sequence may be cloned. The transcript of the non-phage sequence would contain the Operator sequence and the Maturase binding sequence for

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these other bacteriophages such that the non-phage RNA sequence would be encapsidated with Coat Protein and Maturase of the appropriate strain specific phage. In this manner, Armored RNA™ may be composed of Coat Protein and Maturase from any of the RNA bacteriophage species. Similarly to the pAR-2 construct, induction would produce a reRNA which encodes Maturase, Coat Protein, Operator sequence(s), and the Maturase Binding sequence(s) of these other bacteriophages such that the non-bacteriophage RNA sequence would be encapsidated with the Coat Protein and Maturase of the appropriate strain specific phage.

EXAMPLE XII

Plant Virus Armored RNA™

Plant viruses can be used to prepare Armored RNA™. For example, TMV and Potyvirus can be used for this purpose.

1. Tobacco Mosaic Virus Packaging.

It is possible to package recombinant RNA (reRNA) using Tobacco Mosaic Virus (TMV) coat protein in *E. coli* (Hwang 1994a and Hwang 1994b). TMV is a filamentous virus composed of 2,100 of a 17 kDa coat protein that protects a 6.4 kb single stranded, positive sense genomic RNA against ribonucleases. TMV has been used as a model system for studying self-assembly of multimeric biological structures. Under the correct conditions, purified TMV coat protein and the gRNA will spontaneously assemble to form an infectious virus *in vitro*. As well, reRNA packaged *in vitro* with TMV coat protein is protected against ribonuclease digestion (Jupin *et al.*, 1989).

The TMV gRNA has an operator-like sequence called the Origin-of-Assembly-Sequence (OAS) recognized by the coat protein to initiate assembly. The OAS is located at nucleotides 5112-5543 in the wild-type virus, 432 nucleotides in length. The OAS exists as a three stem-loop structure. The minimal length of the OAS required for packaging is 75 nucleotides, nucleotides 5444-5518. The coat protein assembles faster in the 3' to 5' direction along the RNA than in the 5' to 3' direction.

In the TMV-*E. coli* packaging system, TMV coat protein was plasmid encoded and provided *in trans* to the reRNA to be packaged. (Hwang 1994a, Hwang 1994b, U.S. Patent No. 5,443,969). reRNAs, containing a 5' open-reading frame and an OAS, of 1.6

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kb (CAT-OAS RNA) and 2.8 kb (GUS-OAS RNA) were transcribed in *E. coli* from a second plasmid. Co-expression of the coat protein and the reRNA resulted in the packaging of the reRNA. The integrity of the reRNA packaged was assayed by RT-PCR™. Full-length CAT-OAS RNA was detected by RT-PCR™ but full-length GUS-OAS RNA was not detected.

2. Potyvirus Packaging.

Potyvirus is another example of a plant virus that can be used to produce Armored RNA™. Its coat protein has been expressed and assembled into capsids in *E. coli* (Zhao, 1995). However, there was no mention of the RNA which may have been packaged. Presumably, there must be an operator-like sequence as is found for MS2 and TMV. No reference has been found to such a sequence for Potyvirus. A reRNA could be packaged by this coat protein once its operator sequence was discovered.

To define the operator sequence in potyvirus, a systematic set of packaging experiments may be performed, similar to the experiments of Pickett and Peabody (1993). The potyvirus coat protein gene may be expressed from one vector in *E. coli*. Another vector may be used to transcribe different segments of the potyvirus genomic RNA in about 1 kb lengths. These sequences could be fused to a reporter sequence, that is, a known sequence common fused to each segment of the potyvirus genomic RNA. The reporter sequence may be as short as 20 bases. The potyvirus coat protein and the test sequences may be co-expressed in *E. coli* and the viral particles isolated. The RNA may be isolated from the particles and then the RNA assayed for the reporter sequence by one of a number of different methods such as RT-PCR™, Northern blotting, dot blotting, or RPA. Particles containing the reporter sequence would be candidates for containing the operator sequence. The operator sequence can be further defined by performing an iteration of the same experiment, further dividing the 1 kb candidate sequence into 250 base sequences. Once an operator sequence is identified, it can be used with potyvirus coat protein to package heterologous RNA sequences.

By employing a strategy like that used to make bacteriophage Armored RNA™, a recombinant RNA can be transcribed in *E. coli* composed of the potyvirus coat protein sequence, non-potyvirus sequence and the potyvirus operator sequence. This reRNA will be packaged specifically by the potyvirus coat protein because it contains the operator

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sequence at the 3' end. In this system, the coat protein sequence and the operator sequence are *in cis*. Another alternative is to transcribe the coat protein RNA and the non-potyvirus RNA/operator RNA *in trans*. Such a *trans* system was used both with MS2 (Pickett and Peabody, 1993) and with TMV.

EXAMPLE XIII

Animal Virus Armored RNA™

Animal viruses can be used to produce Armored RNA™. An example of this is seen with regard to the alphavirus.

1. Alphavirus Packaging.

Alphaviruses are enveloped positive, single strand RNA viruses which infect mammals, birds, and insects. Examples from this virus family are Semliki Forest Virus (SFV), Sindbis virus, and Venezuelan Equine Encephalitis (VEE). Over the past ten years, these viruses have been adapted for the expression of recombinant RNAs and proteins in animal cells. (Frolov 1996 and U.S. Patent No. 5,217,879)

The alphavirus particle has a single genomic RNA protected by 240 copies of basic capsid protein (C), surrounded by a lipid bilayer containing 240 E1E2 envelope glycoprotein heterodimers. Alphaviruses can infect a variety of cell types and may use more than one receptor to gain entry into a cell. The virus has an icosahedral symmetry. The genomic RNA is capped at the 5' end and it is organized such that the 5' end half encodes non-structural genes for RNA replication and the 3' half encodes the structural genes for packaging the RNA. There is a packaging signal sequence within the non-structural region of the genomic RNA required for efficient encapsidation.

Several strategies have been used to package reRNA using the alphavirus system. In one method, an infectious recombinant virus is produced in which the complete wild-type genomic RNA is maintained and the foreign sequence of interest is inserted 5' or 3' or the structural genes. Foreign RNA sequences up to 2 kb can be packaged. This method was used to package several foreign genes including bacterial chloramphenicol acetyltransferase (CAT) and a truncated form of the influenza hemagglutinin. Cells infected with these recombinant alphaviruses produced the encoded proteins.

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Another strategy for the packaging of reRNA involves the production of non-infectious particles. In this system, the structural and non-structural RNAs are encoded in separate, distinct RNAs with the replicase sequence containing the packaging signal and the foreign RNA sequence. These two RNAs are co-transfected into a cell. The replicase replicates both the non-structural and structural RNAs while the structural RNA generates the proteins which only package the non-structural/foreign gene RNA. These particles are non-infectious because the structural genes are not packaged with the non-structural genes. Using this method, it is estimated that at least 5 kb of foreign RNA can be packaged.

There are other variations of the above described packaging systems involving similar type strategies but most of them are designed to increase the protein expression of the foreign RNA sequence.

The alpha-particles will protect RNA from ribonucleases in much the same manner as the MS2 particles. It is to be expected that the reRNA will be protected from ribonucleases because alphaviruses could not re-infect if their genomic RNA were susceptible to ribonucleases once it was packaged and the viruses were released from their host cell.

The alpha-particles may not be as heat resistant as bacteriophage Armored RNATM, because they have a different coat structure. The bacteriophage Armored RNAs may be the preferred choice for shipping purposes if alphaviruses are heat sensitive. However, any heat sensitivity would not be an insurmountable problem as particles could simply be shipped on dry or wet ice.

Because alphaviruses are physically very similar to HIV, they may be a better pelleting standard for ultra-sensitive assays. The alphaviruses have more in common with HIV and HCV structurally than with the bacteriophages. Thus, they may pellet by centrifugation more like the HIV and HCV they are supposed to mimic. Pelleting the viruses may be important in developing a good standard for an ultrasensitive assay. HIV in 1 ml of plasma can be concentrated by centrifugation and then the RNA is extracted from the viral pellet. Alphavirus particles may also accept longer foreign sequences than the MS2 system, because they have a larger genome than bacteriophage. Alphavirus particles should be stable in serum/plasma for time periods similar to HIV. Many of

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these viruses are transmitted through mosquito vectors. Therefore, the virus must survive exposure to plasma.

EXAMPLE XIV

Construction of Armored DNA

5 There are many well studied single- and double-stranded DNA bacteriophage which infect *E. coli* as well as a number of other bacteria. It is straightforward to clone DNA fragments into a single stranded bacteriophage such as M13 or into the double stranded bacteriophage λ . These recombinant phage are readily purified and quantified by determining the plaque forming units. They may act as standards for PCR™ based assays
10 for DNA viruses. For instance, a λ recombinant bacteriophage containing a conserved DNA sequence of the human pathogen, the Herpes Simplex Virus may act as a double stranded DNA standard. It may be used as a quantitative standard or as a positive control.

EXAMPLE XV

Subtraction of rRNA from total RNA

15 The Armored RNA™ may be used to synthesize large mass amounts of RNA which encodes the antisense sequence of 18S and 28S rRNA. Once the RNA has been isolated and purified, it can be labeled with photobiotin. The biotinylated RNA is added to total RNA in molar excess over the target 18S and 28S rRNA. The mix is heat
20 denatured in a hybridization buffer promoting the formation of RNA hybrids and then allowed to cool to room temperature to encourage the formation of the duplex between the 18S and 28S rRNA with the biotinylated antisense RNA. After hybridization, streptavidin is added to the solution and binds to the biotinylated RNA. A phenol extraction partitions all the unbound streptavidin and biotin/streptavidin complexes into
25 the organic phase. The 18S and 28S rRNA hybridized to the antisense biotinylated RNA also partitions into the organic phase. By this strategy, the 18S and 28S are subtracted from the total RNA. Since the molar quantity of 18S and 28S rRNA is very abundant in

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total, large quantities of antisense RNA would be required for this strategy. Armored RNA™ technology could provide as a means to provide a cheap source of RNA.

EXAMPLE XVI

Construction of RNA size standards

5 Mixtures of RNA of very discrete lengths are often used as RNA size standards for gel electrophoresis. These size standards are used often in Northern blotting to estimate the length of unknown mRNA species. In general, the RNA size standards range from 0.2 to 10 kilobases. The RNA in these standards are susceptible to ribonucleases and they are usually produced by *in vitro* transcription. Therefore, any of the methods
10 described herein may be used to create ribonuclease resistant RNA size standards.

 For example, a series of Armored RNA™ standards can be constructed, each containing an RNA of a different length. DNA fragments of different sizes would be cloned into pAR-1 to construct this series of size standards. The size range of the standards will be dependent on the RNA size limitation of the packaging system. After
15 production, each of the Armored RNA™ size standards would be produced and quantified separately. The standards would be mixed so that the RNA of each of the standards are at the same mass concentration.

 In a preferred embodiment, the mixed Armored RNA™ size standards will be added to a denaturing solution just prior to their use for gel electrophoresis in order to
20 disrupt the RNA-protein complex. The denaturing solution may consist of an acid such as acetic acid, a detergent such as SDS or a chaotrope such as urea. The standards would be heated and then loaded on the gel. The advantage of this embodiment is that there is no chance for the RNA standards to be degraded until just prior to gel electrophoresis.

 In another embodiment, the Armored RNA™ mixture is used as the stock solution
25 from which to isolate the size standards. The RNA may be isolated using a common RNA isolation procedure such as by Chomczynski (1987).

 Of course, it is also possible to isolate the RNA from each of the Armored RNA™ size standards separately and then mix the RNA afterwards.

 Alternatively, it is possible to construct ribonuclease resistant chemically modified
30 RNA size standards, which can be used as a single standard or in sets of mixed standards.

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EXAMPLE XVII**Removal of the non-phage RNA from the phage RNA**

There are applications where a researcher may want an RNA which does not encode any phage sequence. All the Armored RNA™ compositions contain a reRNA which encodes some amount MS2 bacteriophage sequence. One skilled in the art may construct the RNA sequence so that the RNA sequence of interest is flanked by ribozyme sequences as demonstrated previously (Ferré-D'Amaré, 1996). The non-phage RNA may be encapsidated with the ribozyme sequences because these sequences are not very active in *E. coli*. After the RNA is isolated from the Armored RNA™, the RNA may be subjected to buffer conditions which produce optimal ribozyme activity. After ribozyme cleavage, the RNA fragment of interest may be purified by gel electrophoresis or HPLC.

Another method previously used by others involves using Ribonuclease H which cleaves RNA at RNA/DNA duplexes (Lapham, 1996). Oligonucleotides complementary to regions flanking the desired RNA fragment are hybridized to the naked reRNA after it has been purified from the Armored RNA™ particle. This substrate is subjected to RNase H which will cleave the reRNA at the sites where the oligonucleotide has hybridized. DNaseI may be used after to digest all the oligonucleotides.

After purification, the RNA fragment may be capped with RNA guanylttransferase using GTP and SAM which may then be a suitable substrate for translation.

EXAMPLE XVIII**Use of Armored RNA™ to Deliver RNA *In Vitro* and *In Vivo***

Armored RNA™ technology may be used to deliver intact, full length RNA to cells *in vitro* and *in vivo*. Transfection of cells with RNA is performed with naked RNA which is susceptible to ribonucleases. Transient gene expression may be optimized by producing an RNA message as an Armored RNA™, to protect the RNA until it enters a cell. Upon entry in the cell, the protective Coat Protein may be removed by the cellular machinery. The use of a bacteriophage system to deliver intact RNA would be similar to a TMV system which was previously employed. A recombinant RNA was packaged *in*

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vitro using TMV Coat Protein and successfully delivered into plant cells and into frog cells (Gallie et al, 1987).

The RNA need not be capped for translation. An encephalomyocarditis virus or Polio translational leader sequence may be fused upstream of the coding sequence to promote the internal initiation of translation *in vitro* or *in vivo* (Elroy-Stein, 1989). This system is cap independent for translation. The Armored RNA™ may be mixed with DOTAP, Lipofectin, Transfectam or Lipofectamine to form cationic liposomes to optimize membrane fusion. Liposomes have been used successfully to increase the efficiency of RNA transfection with cells from tissue culture (Lu, 1994; Dwarki, 1993). As well, the Maturase or the Coat Protein could be genetically engineered so that either or both contain a peptide sequence which recognize specific cell surface receptors. These ligands would promote tissue-specific uptake of the Armored RNA™. Peptide sequences up to 24 amino acids can be inserted into the MS2 Coat Protein such that the recombinant Coat Protein will maintain its ability to assemble into capsids (Mastico et al., 1993). Peptide sequences which recognize cell-specific receptors could be incorporated into Coat Protein. The recombinant Coat Proteins would be used to package reRNA, *in vitro* or *in vivo*, into Armored RNA to produce a multivalent macromolecule which would recognize specific cell surface receptors. The multivalent property of the Armored RNA would confer a strong affinity for the cell surface receptor. The peptide receptor sequences could be derived using phage display technology (Barry, 1996; Pasqualini, 1996) or by using previously characterized peptide sequences (Hart, 1994).

An mRNA may be packaged *in vivo* by cloning the gene of interest, such as the CEA gene (associated with many different tumors and thought to be a tumor rejection antigen), into a vector such as pAR-1. The gene would be cloned immediately downstream of the Operator sequence in pAR-1. As well, the EMCV translation sequence would be cloned between the Operator and the CEA coding sequence. In this way, the CEA RNA may be translated without requiring a 5' CAP to enhance translation of the mRNA.

For *in vitro* transfection, the CEA-Armored RNA™ would be mixed with the tissue cultured cells, with or without forming liposomes before transfection. The liposomes may enhance fusion of the Armored RNA™ with the cells. Also, the CEA-

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Armored RNA™ may be microinjected into oocytes. The use of Armored RNA™ would forgo the requirement for the usual precautions in handling RNA. In these procedures, it is expected that the Armored RNA™ would dissociate upon entering the target cell and release their packaged RNA so that it is available for translation.

5 *In vivo*, the Armored RNA™ may be injected into tissues by a variety of routes such as intravenous or intramuscular to produce the protective immune response. It would be desirable that the Armored RNA™ could be taken up by the appropriate immune cells to produce the strongest and most protective immune response. One skilled in the art could introduce the appropriate counter receptor (or ligand) sequence into the
10 Maturase or Coat Proteins so that the Armored RNA™ would bind and be taken up by the strongest immunomodulators. This strategy would require prior knowledge about the some specific cell specific receptors on these immunomodulators so that they could be targeted appropriately.

 If a one time vaccination is not enough to produce a protective immune response,
15 then multiple immunizations may be required. In this case, the Armored RNA™ themselves may be immunogenic and would become less effective at transfection as the individual develops immunity to the Armored RNA™ itself. Multiple vaccinations may involve using Armored RNA™ developed from RNA bacteriophage from several different serotypes. All of the Armored RNA™ vaccinations would contain the same protective
20 mRNA, however, it would be packaged by phage capsids from different serotypes. For instance, the RNA coliphages are divided into serological groups I, II, III and IV. There are also the two strains of *P. aeruginosa* RNA bacteriophage. Thus, the CEA mRNA may be packaged in as many as 6 different Armored RNA™ and each would be used for each different immunization.

25 A capped mRNA may be encapsidated co- or post-transcriptionally. An mRNA with a 5' cap may be synthesized by using a cap analog nucleotide during *in vitro* transcription or an RNA transcript may be capped with RNA guanylttransferase using GTP and S-adenosyl methionine as substrates. After mRNA synthesis, the mRNA may be mixed with Coat Protein and/or Maturase to produce Armored RNA™. The mRNA may
30 contain one or more Operator sequences and/or one or more Maturase Binding sites but they are not essential for the packaging of long RNA molecules. These Armored RNA™

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synthesized completely by *in vitro* methodologies may be used for transfection into cells. Again, the Coat Protein may be manipulated to express a ligand which will enhance the binding of the Armored RNA™ to the target cell and promotes its uptake and processing.

Recently, phage display technology was used to select for peptides which bound selectively to specific cell types *in vitro* (Barry, 1996) and *in vivo* (Pasqualini, 1996). Libraries of filamentous DNA bacteriophage displaying random peptide sequences were either panned over mouse fibroblast cells grown in culture or they were injected intravenously into mice and the bound bacteriophage were eluted from different organs. Panning over the mouse fibroblast cells resulted in bacteriophage clones which bound well to fibroblast, hepatoma, and mastocytoma cells but not to myoblast or macrophage cells. Intravenous injection into a mouse resulted in bacteriophage which bound selectively to brain tissue and to kidney. Peptides were synthesized based on the sequence derived from the bacteriophage which bound selectively to brain. These peptides were able to block the adherence of the bacteriophage to the brain capillaries. These data suggest that Armored RNA™ can be developed into a delivery system for gene therapy or drug treatment which will target specific organs. Indeed, it has been demonstrated that filamentous bacteriophage displaying the peptide sequence RGD, which promotes binding to the cell surface receptor integrin, are internalized in human HEp-2 cells (Hart, 1994).

The Armored RNA™ may also be added to *in vitro* translation systems such as a rabbit reticulocyte extract. As stated earlier, capped, polyA mRNA may be used if the reRNA is packaged post-transcription using an *in vitro* packaging system.

The Coat Protein or the Maturase could be modified to contain the biotinylation peptide sequence which leads to this peptide becoming biotinylated in *E. coli*. In this strategy, the Armored RNA™ would be coated on the surface with biotin molecules which are accessible to streptavidin. Streptavidin may be conjugated to ligands which recognize specific cell surface receptors. The ligand-streptavidin conjugate may be incubated with the Armored RNA™ to coat it with the streptavidin-ligand conjugate. The ligand-streptavidin-Armored RNA™ complex may be used to deliver the packaged RNA.

Antibodies to Armored RNA™ could be conjugated to receptor molecules as previously performed (Douglas et al., 1996). The Armored RNA™ particles would be pre-coated with an antibody-receptor conjugate thus conferring receptor specificity to the

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Armored RNA™. The Armored RNA™ Antibody Receptor complex would bind to a specific cell and then be taken into the cell.

Armored RNA™ may be made more effective as a delivery vector if the Armored RNA™ disassembles easily once it enters the cell. By using mutant forms of the Coat Protein in the Armored RNA™, it may be possible to produce less stable structures which may protect the encapsidated reRNA from ribonucleases but dissociates readily upon entry into a cell. Mutant forms of the Coat Protein have been studied which are less efficient at capsid assembly (LeCuyer, 1995) and have less thermal stability compared to the wild-type Coat Protein (Stonehouse, 1993). Higher concentrations of the Coat Protein are needed to induce capsid assembly than for the wild-type sequence. However, Armored RNA™ assembled *in vivo* in the presence of these mutant Coat Proteins may be more efficient for RNA delivery.

EXAMPLE XIX

Production of RNA to construct an affinity column

The need for RNA extends to producing columns for the affinity purification of proteins which bind to specific RNA sequences. Such proteins are involved in the regulation of translation and in RNA turnover. Armored RNA™ technology may be used to produce large quantities of a specific RNA sequence. The reRNA would be purified from the Armored RNA™ particles. The reRNA could be covalently conjugated to a solid phase resin. Alternatively, a more generic resin may be developed in which Coat Protein was covalently bound to the resin. The reRNA could be immobilized to the resin by its binding to the Coat Protein through the Operator sequence. The non-phage sequence in the reRNA would then be used to pull out proteins which specifically recognize it.

In another format, the protein-reRNA binding could occur in solution, to better mimic the actual interaction and then the free reRNA and the protein-reRNA complexes would be concentrated using a Coat Protein column. The column may be subjected to ribonuclease to release the proteins which bound to the reRNA. These proteins may then be characterized by gel electrophoresis or the material eluted from the column may be injected into animals such as mice and rabbits to produce antibodies. Once antibodies are

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produced, then cDNA expression libraries may be screened for the gene responsible for the protein binding to the RNA.

EXAMPLE XX

Packaging of Capped mRNA *in vivo*

5 Using Armored RNA™ technology, mRNA may be capped and packaged in *E. coli*. The enzyme RNA guanyltransferase is responsible for capping the 5' terminus of an mRNA using GTP and S-adenosylmethionine (SAM) as substrates. RNA guanyltransferase has been cloned and expressed in *E. coli* in an active form (Cong, 1995). The RNA guanyltransferase may be co-expressed with the RNA to be capped and
10 to be packaged as an Armored RNA™ particle. In the preferred embodiment, the non-phage RNA would be positioned at the 5' end of the reRNA.

EXAMPLE XXI

Encapsulation as a Method For Producing Nuclease-Resistant RNA

15 "Caging," or encapsulation of RNA makes an RNA not accessible to an RNase-containing solution. Encapsulation of RNA by viral proteins is an example of microencapsulation. Microencapsulation is a process whereby a molecule (in this case RNA) is surrounded by a structure that denies access to the internalized molecule. Microencapsulation confers nuclease-resistance upon RNA by sequestering the RNA from the nuclease-contaminated environment. In addition to viral protein encapsidation, the
20 most common methods of microencapsulation are internalization in liposomes and inclusion in polymer matrices. Viral protein encapsidation has proven capable of providing nuclease-resistant RNAs (see Armored RNA above). Liposomes and polymer matrices partition molecules in a manner that is analogous to protein encapsidation, thus they are expected to perform equally well in protecting internalized RNA standards.

25 1. Liposomes

 The formation of liposomes occurs spontaneously when lipids are dispersed in aqueous solutions. Reagents that are present in the aqueous solution are encapsulated during liposome formation, providing a relatively simple method for microencapsulating

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pharmaceuticals, cells, proteins, and nucleic acids. Several methods exist for producing liposomes (Vemuri 1995). The best characterized of these involves adding an excess volume of reagent-containing aqueous buffer to a round-bottomed flask in which a lipid solution had been lyophilized. The addition of the aqueous solution first dissolves the lipids and then causes them to form liposomes that engulf portions of the aqueous solution. This effectively produces liposome-encapsulated reagents that can be purified, quantified, stored, and eventually used in diagnostic assays.

2. Polymer matrices

A variety of monomers can be converted to polymeric microspheres by dropwise dispersion into a solution that encourages polymerization. Molecules dissolved in either the monomer solution or the solution in which the polymerization is occurring will be trapped in the microspheres. Purification of the microspheres provides trapped reagents that are inaccessible to macromolecules in the surrounding solution.

An illustration of how to produce reagent-containing microspheres comes from a study on the microencapsulation of rotavirus in alginate/spermine matrices (Offit 1994). A rotavirus suspension mixed with sodium alginate (0.68 mM) was dispersed as 5 μ m droplets into 0.55 mM spermine hydrochloride. The mixture was centrifuged at 600 x g and washed several times to purify the rotavirus containing microspheres. Analysis of the microspheres revealed that the rotavirus were contained within the polymer matrix, and that upon release, they were still infectious. These two points are important as it reveals that microspheres sequester molecules from the surrounding solution and that the internalized molecule is being maintained in its native state. Microencapsulation of an RNA standard could be done by replacing the rotavirus suspension with an RNA solution.

A single, general method can be employed for incorporating microencapsulated RNAs into a diagnostic assay. RNAs possessing domains sufficient for detection (primer binding or hybridization sites) would be encapsulated by one of the methods mentioned above. The encapsulated RNA would be purified, quantified, and stored for future use. For diagnostics, the microencapsulated standard would be diluted and aliquoted into the samples at an appropriate step. If the standard RNA isolation procedure in the diagnostic protocol does not adequately release the standard, or if the standard is added after the isolation step, then heating the sample to 90°C for 2-5 minutes will be sufficient for

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release from most microcapsules. The RNA detection portion of the assay could then be performed.

EXAMPLE XXII

Noncovalently Bound RNA as a Nuclease-Resistant Standard

5 RNAs are bound with relatively high affinity by a variety of molecules. In many cases, the RNA can be completely coated by the interacting molecules. There are examples whereby the presence of the bound molecules renders the RNA resistant to nuclease degradation. This general scheme provides an additional method for generating nuclease-resistant RNA standards.

10 There are a variety of proteins, nucleic acids, and small molecules that bind RNA. Footprinting experiments with HIV-1 nucleocapsid protein (Allen 1996) and the regA protein of T4 (Winter *et al.* 1987) show that bound molecules can render an RNA resistant to nucleases. Proteins and small molecules that bind RNA cooperatively often coat large regions of the polymers. Examples include the T4 gene 32 protein (von Hippel 15 1982), MS2/R17 coat protein (Witherall 1990), and the small molecules spermine and spermidine (McMahon 1982). The combination of cooperative binding and nuclease occlusion can effectively protect large domains of RNAs from degradation (Allen 1996) making them ideal nuclease-resistant standards. Poly-L-lysine is able to protect the natural double-stranded RNAs interferon inducers, larifan and ridostin, from ribonucleases in 20 human blood serum. The free interferon inducers in serum completely lost their activity after 4 hour incubation in serum. The protective activity of poly-L-lysine increased in parallel with the increase in its molecular weight. The protective activity was maximal with material of 12,300 +/-1,000 Daltons (Surzhik *et al.*, 1993).

25 Cetyltrimethylammonium bromide (CTAB) is a cationic detergent which has been long used for the isolation of nucleic acids by virtue of its ability to bind to and precipitate nucleic acids but not protein (Jones, 1953; Jones, 1963). The positively charged head groups can bind to the negatively charged backbone of the RNA, protecting the RNA from ribonuclease. Experiments by Winkler and Kessler (unpublished) demonstrate that 20 mM CTAB protects as much as 5 micrograms RNA from digestion by RNase A and

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RNase T1. If 0.5% Triton X-100 is added to the CTAB/RNA solution, the CTAB is solubilized by the Triton X-100 and the protective action is reversed.

Similarly, nucleic acids afford nuclease protection by directly interacting with RNA (Weigand 1975). Short (ten nucleotides), random sequence nucleic acids can be hybridized to an RNA, effectively coating the molecule. Incubation in a sample that includes RNases would not be detrimental, as the RNA would be bound by the randomers, and thus protected. If an amplification scheme is used for detection, the short randomers could serve as primers for reverse transcriptase.

A general scheme can be devised by one of skill for employing any of the noncovalently bound RNAs as standards. An RNA with sequences for primer binding (for RT-PCR, NASBA, or 3SR) or probe hybridization (branched DNA assay or DiGene's antibody based assay) can be prebound by any of the RNA binding molecules mentioned in the preceding paragraphs. This bound RNA would be accurately quantified and stored for future use. The bound RNA would be diluted, and aliquoted into samples, either before or after RNA purification. Prior to amplification or hybridization (depending on the detection protocol), the sample would be heated to 90°C to release the RNA standard. The amplification or hybridization could then be performed, producing a signal for detection.

EXAMPLE XXIII

RNA Bound by gp32 is Nuclease-Resistant

An RNA bound by the protein encoded by gene 32 of the bacteriophage T4 (gp32) was used to demonstrate the effectiveness of noncovalent binding methodology for making RNA standards. gp32 is known to bind RNA and ssDNA cooperatively (von Hippel 1982). The purified protein (~1 μ M) was mixed with a radiolabeled RNA target (~100 nM) for five minutes at room temperature in a buffer consisting of 50 mM HEPES pH 7.4, 200 mM NaCl, and 2.5 mM MgCl₂. By gel shift assay, approximately 50% of the RNA molecules were bound by the protein. A tenth volume of goat serum was added to the coated RNA and the mixture was incubated at 25°C for ten minutes. The RNA was separated via PAGE and detected by autoradiography. The amount of intact RNA was greater than an identical RNA sample that lacked the protein. This illustrates that the

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presence of gp32 protects RNA from serum-mediated degradation and argues that the concept of nuclease protection generated by cooperatively bound molecules is valid.

A sample of the same RNA used above was bound by gp32 using the described procedure. This sample was heated to 95°C for 2 minutes and then reverse transcribed by AMV reverse transcriptase using a primer specific to the 3' end of the RNA. The resulting cDNA was serially diluted and the various dilutions were used to initiate a standard PCR reaction using primers specific to the RNA standard. The PCR products were analyzed by gel electrophoresis. The dilution at which product was first observed was the same for the gp32-bound RNA as for an unbound RNA subjected to the same procedure. This indicates that the T4 protein does not affect the capacity of RT to use the RNA as a template, nor does it subsequently affect the PCR reaction. Protein-coated RNAs have also been diluted into a 10% serum solution for fifteen minutes and then applied to an RT-PCR experiment. Products of the appropriate size were generated, providing evidence that cooperative binding of an RNA is a useful method for producing stable RT-PCR controls.

EXAMPLE XXIV

Protection of RNA through Chemical Modification

One method for making RNAs resistant to enzyme-mediated degradation is to chemically alter the RNA so that the mechanism for degradation is blocked or so that RNases no longer recognize the molecule as RNA. Such alterations can be made to nucleotides prior to their incorporation into RNA or to RNA after it has been formed. Ribose (Piecken 1991) and phosphate (Black 1972) modifications have been shown to enhance RNA stability in the presence of nucleases. Modifications of the 2' hydroxyl and internucleotide phosphate confers nuclease resistance by altering chemical groups that are necessary for the degradation mechanism employed by ribonucleases (Heidenreich 1993). Another method could be accomplished by chemically adding molecules (e.g., carbohydrates) to the RNA standard. These chemical groups could be removed enzymatically or chemically during the detection protocol to allow for reverse transcription. Provided that they are templates for the reverse transcription or hybridization protocols used in nucleic acid detection schemes, chemically modified RNAs

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will be ideally suited as RNA standards as they are considerably more stable than non-modified RNA.

The general scheme for using chemically modified RNAs as standards in diagnostic assays is to simply replace the current RNA standards with modified RNAs possessing the same sequence. Transcribe the DNA template encoding the RNA standard using T7 RNA polymerase, replacing the standard NTPs with ribose or phosphate modified NTPs. The resulting full-length RNA can be purified, quantified and stored for future use. At the time of the assay, the modified RNA standard can be appropriately diluted, aliquoted into sample either before or after RNA purification, and then co-detected with the sample RNA.

EXAMPLE XXV

Protection of RNA With 2' Fluoro CTP and UTP

To test the usefulness of modified RNAs as standards, nucleotide analogs were incorporated into transcripts and compared to unmodified RNAs. 2' fluoro CTP and UTP were incorporated into transcripts by 0.5 units/ μ l of T7 RNA polymerase in transcription buffer (40 mM Tris pH 8.0, 20 mM NaCl, 6 mM $MgCl_2$, 6 mM $MnCl_2$, 2 mM spermidine, 10 mM DTT). Ten picomoles of modified and unmodified RNA were reverse transcribed by AMV reverse transcriptase. A dilution series of the resulting cDNAs were amplified by PCR. The products were separated via PAGE and analyzed by ethidium bromide staining. Both the modified and unmodified RNA samples generated identical results, indicating that the modifications have no effect on the quantification of the RNAs by RT-PCR. The modified RNAs were incubated in serum for one hour and the products were size-separated by PAGE. No degradation of the modified RNAs was apparent, while an unmodified RNA of the same sequence was completely degraded. Thus, the modified RNAs would be far superior to unmodified RNA as they could be added earlier in the diagnostic protocol (providing a better internal standard) and they would be more stable during storage.

The ultimate test of the modified RNA is to show its compatibility with an existing diagnostic assay. A template encoding the sequence of the standard for the Amplicor HIV Monitor™ assay was transcribed with 2'F CTP and UTP as above. A series of

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dilutions of the 2' F modified RNA was applied to the Amplicor HIV Monitor™ test. The modified standard produced a signal in a concentration dependent fashion, providing evidence that the modified RNA was being reverse transcribed and that the resulting cDNA was being PCR amplified. The modified RNA standard was then compared to an unmodified RNA. Both standards produced approximately equal signals when introduced after the lysis step of the detection protocol, but when the two RNAs were incubated in plasma for fifteen minutes prior to incorporation into the detection protocol, the signal produced by the unmodified RNA was indistinguishable from background whereas the modified RNA produced signals that were nearly equivalent to that observed when the RNA was not preincubated in plasma. This latter observation points to the obvious advantage of the modified RNA standard, namely that the RNA can be added earlier in the protocol (prior to sample lysis) providing a better control for the overall experiment. Additionally, nuclease-resistance will be beneficial during storage, as the likelihood of degradation prior to assaying will be greatly diminished.

Phosphate modified nucleotides, in the form of α -thiols of CTP and UTP, were incorporated into transcripts by T7 RNA polymerase as the 2'F above. The resulting RNAs were templates for reverse transcriptase. PCR analysis revealed that the modified RNAs were converted to cDNAs as efficiently as unmodified RNAs.

Although the work described used only modified pyrimidines, modified purines can also be incorporated into RNA [Aurup *et al.* (1994)]. This might be important in a case where a high concentration of a purine-specific nuclease is present in a group of samples. Because a variety of nucleotide analogs exist that can be incorporated, RNAs can be tailored for different storage and reaction conditions to ensure optimum stability and assay efficiency.

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EXAMPLE XXVI

Assay for Ribonuclease Protection

To test a potential ribonuclease-resistant construct for ribonuclease resistance, a protected RNA construct is incubated with ribonuclease, such as Ribonuclease 1 or Ribonuclease A, at 37°C for several different time points, 15 minutes, 30 minutes, 1 hour, 2 hours. During the same experiment, naked RNA isolated from the same Armored RNA

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construct is also incubated with the ribonuclease. After each time period, the ribonuclease is inactivated by phenol extraction. The phenol also strips the coat protein from the RNA in an Armored RNA construct. After phenol extraction, the RNA is ethanol precipitated. The isolated RNA is fractionated on a denaturing formaldehyde agarose gel, stained with ethidium bromide and assessed for degradation. Degradation is assessed by comparing the ribonuclease treated samples to RNA which was not ribonuclease treated.

Alternatively, instead of analyzing the RNA by gel electrophoresis, a sample can be subjected to competitive RT-PCR to obtain a more accurate, quantitative result. The use of PCR also has the advantage of detecting small amounts of material.

EXAMPLE XXVII

Standard Steps in Using Any RNase Resistant Standard

Many assays employing RNase resistant standards will have the same basic format as the Amplicor HIV Monitor™ assay described above. In such a kit, known amounts of RNase resistant standard is added to the RNA sample. The sample RNA and the RNA standard (eg., Armored RNA™, chemically modified RNA or any other ribonuclease protected RNA) are co-purified from plasma or from cells. For some assays, the sample RNA will already have been purified. In such cases, the RNase resistant standard may be added directly to the sample RNA. The mixture can then be heated for 5 minutes, 70°C to strip off a protein coat, or any other molecule that confers ribonuclease resistance, or treated in any other manner necessary.

After the RNA has been purified or heated, the sample RNA and the standard RNA are reverse transcribed and then co-amplified by PCR. If the standard RNA has a deletion or insertion, then the PCR products may be fractionated on a gel. The PCR product generated from the RNA standard will have a different mobility than the PCR product derived from the sample RNA. A standard curve can be generated plotting the concentration of RNA standard and the amount of PCR product generated from the standard. The concentration of the sample RNA is derived by interpolation within the linear range of the curve.

In the HIV Monitor™ assay, the RNA standard has a 26 nucleotide substitution compared to the sample RNA sequence. Quantification of the PCR products are derived

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by using immobilized oligonucleotides to specifically capture the PCR products by hybridizing to the test PCR product or the standard PCR product. The PCR products are 5' biotinylated. The captured PCR products are detected colorimetrically using streptavidin horseradish peroxidase. Once again, the concentration of the unknown RNA sample is calculated using the signal produced by the known concentration of the standard RNA.

* * *

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions, methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

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(A) APPLICATION NUMBER: US 08/675,153
(B) FILING DATE: 03-JUL-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTTTTCGGGG TCCTGCTCAA CTT

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(2) INFORMATION FOR SEQ ID NO: 2:

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- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GATTAGATCT GAGTTGAACT TCTTTGTTGT CTC

34

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1718 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCATGGCTAT CGCTGTAGGT AGCCGGAATT CCATTCCTAG GAGGTTTGAC CTGTGCGAGC	60
TTTGTAGTACC CTTGATAGGG AGAACGAGAC CTTGCTCCCC TCCGTTCCGG TTTACGCGGA	120
CGGTGAGACT GAAGATAACT CATTCTCTTT AAAATATCGT TCGAACTGGA CTCCCGGTCG	180
TTTAACTCG ACTGGGGCCA AAACGAAACA GTGGCACTAC CCCTCTCCGT ATTCACGGGG	240
GGCGTTAAGT GTCACATCGA TAGATCAAGG TGCCTACAAG CGAAGTGGGT CATCGTGGGG	300
TCGCCCCGTAC GAGGAGAAAG CCGGTTTCGG CTTCTCCCTC GACGCACGCT CCTGCTACAG	360
CCTCTTCCCT GTAAGCCAAA ACTTGACTTA CATCGAAGTG CCGCAGAACG TTGCGAACCG	420
GGCGTCGACC GAAGTCCTGC AAAAGGTCAC CCAGGGTAAT TTTAACCTTG GTGTTGCTTT	480
AGCAGAGGCC AGGTCGACAG CCTCACAACG CGCGACGCAA ACCATTGCGC TCGTGAAGGC	540
GTACACTGCC GCTCGTCGCG GTAATTGGCG CCAGGCGCTC CGCTACCTTG CCCTAAACGA	600
AGATCGAAAG TTTCGATCAA AACACGTGGC CGGCAGGTGG TTGGAGTTGC AGTTCGGTTG	660
GTTACCACTA ATGAGTGATA TCCAGGGTGC ATATGAGATG CTTACGAAGG TTCACCTTCA	720
AGAGTTTCTT CCTATGAGAG CCGTACGTCA GGTCGGTACT AACATCAAGT TAGATGGCCG	780
TCTGTCGTAT CCAGCTGCAA ACTTCCAGAC AACGTGCAAC ATATCGCGAC GTATCGTGAT	840
ATGGTTTTAC ATAAACGATG CACGTTTGGC ATGGTTGTGC TCTCTAGGTA TCTTGAACCC	900
ACTAGGTATA GTGTGGGAAA AGGTGCCTTT CTCATTGCTT GTCGACTGGC TCCTACCTGT	960
AGGTAACATG CTCGAGGGCC TTACGGCCCC CGTGGGATGC TCCTACATGT CAGGAACAGT	1020
TACTGACGTA ATAACGGGTG AGTCCATCAT AAGCGTTGAC GCTCCCTACG GGTGGACTGT	1080

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GGAGAGACAG GGCACCTGCTA AGGCCCAAAT CTCAGCCATG CATCGAGGGG TACAATCCGT 1140
 ATGGCCAACA ACTGGCGCGT ACGTAAAGTC TCCTTTCTCG ATGGTCCATA CCTTAGATGC 1200
 GTTAGCATT AATCAGGCAAC GGCTCTCTAG ATAGAGCCCT CAACCGGAGT TTGAAGCATG 1260
 GCTTCTAACT TTAATCAGTT CGTTCTCGTC GACAATGGCG GAACTGGCGA CGTGAATGTC 1320
 GCCCCAAGCA ACTTCGCTAA CGGGGTCGCT GAATGGATCA GCTCTAACTC GCGTTCACAG 1380
 GCTTACAAAG TAACCTGTAG CGTTCGTCAG AGCTCTGCGC AGAATCGCAA ATACACCATC 1440
 AAAGTCGAGG TGCCTAAAGT GGCAACCCAG ACTGTTGGTG GTGTAGAGCT TCCTGTAGCC 1500
 GCATGGCGTT CGTACTTAAA TATGGAATA ACCATTCCAA TTTTCGCTAC GAATTCGGAC 1560
 TGCGAGCTTA TTGTTAAGGC AATGCAAGGT CTCCTAAAAG ATGGAAACCC GATTCCCTCA 1620
 GCAATCGCAG CAAACTCCGG CATCTACTAA TAGACGCCGG CCATTCAAAC ATGAGGATTA 1680
 CCCATGTCGA AGACAACAAA GAAGTTCAAC TCAGATCT 718

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GATTGGTACC TGCTATGTCA GTTCCCCTTG GTTCTCT 37

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 41 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GATTAGATCT AAGTTGGAGG ACATCAAGCA GCCATGCAAA T 41

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1867 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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CCATGGCTAT CGCTGTAGGT AGCCGGAATT CCATTCTCTAG GAGGTTTGAC CTGTGCGAGC 60
TTTTAGTACC CTTGATAGGG AGAACGAGAC CTTCTGCCCC TCCGTTTCGCG TTTACGCGGA 120
CGGTGAGACT GAAGATAACT CATTCTCTTT AAAATATCGT TCGAACTGGA CTCCCGGTCTG 180
TTTAACTCG ACTGGGGCCA AAACGAAACA GTGGCACTAC CCCTCTCCGT ATTCACGGGG 240
GGCGTTAAGT GTCACATCGA TAGATCAAGG TGCCTACAAG CGAAGTGGGT CATCGTGGGG 300
TCGCCCCGTAC GAGGAGAAAAG CCGGTTTCGG CTTCTCCCTC GACGCACGCT CCTGCTACAG 360
CCTCTTCCCT GTAAGCCAAA ACTTGACTTA CATCGAAGTG CCGCAGAACG TTGCGAACCG 420
GGCGTCGACC GAAGTCCTGC AAAAGGTCAC CCAGGGTAAT TTTAACCTTG GTGTTGCTTT 480
AGCAGAGGCC AGGTCGACAG CCTCACAACCT CGCGACGCAA ACCATTGCGC TCGTGAAGGC 540
GTACACTGCC GCTCGTCGCG GTAATTGGCG CCAGGCGCTC CGCTACCTTG CCCTAAACGA 600
AGATCGAAAG TTTGATCAA AACACGTGGC CGGCAGGTGG TTGGAGTTGC AGTTCGGTTG 660
GTTACCACTA ATGAGTGATA TCCAGGGTGC ATATGAGATG CTTACGAAGG TTCACCTTCA 720
AGAGTTTCTT CCTATGAGAG CCGTACGTCA GGTCTGGTACT AACATCAAGT TAGATGGCCG 780
TCTGTCTAT CCAGCTGCAA ACTTCCAGAC AACGTGCAAC ATATCGCGAC GTATCGTGAT 840
ATGGTTTTAC ATAAACGATG CACGTTTGGC ATGGTTGTG TCTCTAGGTA TCTTGAACCC 900
ACTAGGTATA GTGTGGGAAA AGGTGCCTTT CTCATTCTGT GTCGACTGGC TCCTACCTGT 960
AGGTAACATG CTCGAGGGCC TTACGGCCCC CGTGGGATGC TCCTACATGT CAGGAACAGT 1020
TACTGACGTA ATAACGGGTG AGTCCATCAT AAGCGTTGAC GCTCCCTACG GGTGGACTGT 1080
GGAGAGACAG GGCACTGCTA AGGCCCAAAT CTCAGCCATG CATCGAGGGG TACAATCCGT 1140
ATGGCCAACA ACTGGCGCGT ACGTAAAGTC TCCTTTCTCG ATGGTCCATA CCTTAGATGC 1200
GTTAGCATT ATCAGGCAAC GGCTCTCTAG ATAGAGCCCT CAACCGGAGT TTGAAGCATG 1260
GCTTCTAACT TTAATCAGTT CGTTCTCGTC GACAATGGCG GAACTGGCGA CGTGACTGTC 1320
GCCCCAAGCA ACTTCGCTAA CGGGGTCGCT GAATGGATCA GCTCTAACTC GCGTTCACAG 1380
GCTTACAAAG TAACCTGTAG CGTTCGTCAG AGCTCTGCGC AGAATCGCAA ATACACCATC 1440
AAAGTCGAGG TGCTTAAAGT GGCAACCCAG ACTGTTGGTG GTGTAGAGCT TCCTGTAGCC 1500
GCATGGCGTT CGTACTTAAA TATGGAACCTA ACCATTCCAA TTTCGCTAC GAATCCGAC 1560
TGCGAGCTTA TTGTTAAGGC AATGCAAGGT CTCCTAAAAG ATGGAAACCC GATTCCCTCA 1620
GCAATCGCAG CAAACTCCGG CATCTACTAA TAGACGCCGG CCATTCAAAC ATGAGGATTA 1680
CCCATGTCGA AGACAACAAA GAAGTTCAAC TCAGATCTAA GTTGGAGGAC ATCAAGCAGC 1740

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CATGCAAATG TTAAAACATA GCACTATAGA ACTCTGCAAG CCTCGAGTGA GAGTGCATCC 1800
AGTGCAATGCA GGGCCTATTG CACCAGGCCA GATGAGAGAA CCAAGGGGAA CTGACATAGC 1860
AGGTACC 1867

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCAGAAAGCG TCTAGCCATG GCGT

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(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTCGCAAGCA CCCTATCAGG CAGT

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CLAIMS

1. A nucleic acid standard comprising a nuclease resistant nucleic acid segment
5 comprising a sequence coding a standard nucleic acid.

2. The nucleic acid standard of claim 1, wherein the nucleic acid standard is further
defined as an RNA standard comprising a ribonuclease resistant RNA segment comprising
10 a sequence coding a standard RNA.

3. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment is
caged in a ribonuclease resistant structure.
15

4. The RNA standard of claim 3, wherein the ribonuclease resistant structure is
comprised of a Viral Coat protein.
20

5. The RNA standard of claim 4, wherein the viral Coat Protein is a modified viral
Coat Protein.

6. The RNA standard of claim 4, wherein the viral Coat Protein is a bacteriophage
viral Coat Protein.
25

7. The RNA standard of claim 6, wherein the bacteriophage viral Coat Protein is of
an *E. coli* bacteriophage of genetic subclass A or B
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8 The RNA standard of claim 7, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage of genetic subclass A.

5 9. The RNA standard of claim 7, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage in serological group I, II, II, or IV.

10 10. The RNA standard of claim 9, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage in serological group I.

15 11. The RNA standard of claim 4, wherein the bacteriophage viral Coat Protein is of an MS2/R17 bacteriophage.

20 12. The RNA standard of claim 4, wherein the bacteriophage viral Coat Protein is of a *Pseudomonas aeruginosa* RNA bacteriophage.

25 13. The RNA standard of claim 12, wherein the bacteriophage viral Coat Protein is of a *Pseudomonas aeruginosa* PRR1 or PP7 bacteriophage

30 14. The RNA standard of claim 4, wherein the bacteriophage viral Coat Protein is of a filamentous bacteriophage.

15. The RNA standard of claim 4, wherein the viral Coat Protein is a viral Coat Protein of a plant or animal virus.

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16. The RNA standard of claim 15, wherein the viral Coat Protein is a Coat Protein of a tobacco mosaic virus, a feline immunodeficiency virus, or an alphavirus.

5 17. The RNA standard of claim 4, wherein the ribonuclease resistant RNA segment is bound to a viral Maturase protein.

10 18. The RNA standard of claim 17, wherein the viral Maturase protein is a modified viral Maturase protein.

15 19. The RNA standard of claim 3, wherein the ribonuclease resistant structure is comprised of lipids.

20 20. The RNA standard of claim 19, wherein the ribonuclease resistant structure is a liposome.

21. The RNA standard of claim 3, wherein the ribonuclease resistant structure is a synthetic microcapsule.

25 22. The RNA standard of claim 21, wherein the ribonuclease resistant structure is a polymer matrix.

30 23. The RNA standard of claim 22, wherein the polymer matrix is agarose or acrylamide.

24. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises an Operator coding sequence and a viral Maturase protein binding site.

5 25. The RNA standard of claim 24, wherein the Operator coding sequence is a modified Operator coding sequence.

10 26. The RNA standard of claim 24, wherein viral Maturase protein binding site is a modified viral Maturase protein binding site.

15 27. The RNA standard of claim 24, wherein the Maturase protein binding site is included in a viral Maturase protein coding sequence.

28. The RNA standard of claim 24, wherein the ribonuclease resistant RNA segment further comprises a viral Maturase protein coding sequence.

20 29. The RNA standard of claim 28, wherein the Maturase protein coding sequence codes a modified viral Maturase protein.

25 30. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment is a chemically modified ribonuclease resistant RNA.

30 31. The RNA standard of claim 30, wherein the chemically modified ribonuclease resistant RNA is comprised of chemically modified nucleotides.

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32 The RNA standard of claim 30, wherein the chemically modified ribonuclease resistant RNA is prepared by chemical modification of a previously transcribed RNA transcript.

5

33. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment is coated with a ribonuclease resistant coating.

10

34. The RNA standard of claim 33, wherein the ribonuclease resistant coating is comprised of a protein.

15

35. The RNA standard of claim 34, wherein the protein is bound in a sequence independent manner to the ribonuclease resistant RNA.

20

36. The RNA standard of claim 35, wherein the protein is MS2/R17 coat protein, HIV-1 nucleocapsid protein, gp32 of bacteriophage T4, or the regA protein of T4.

25

37. The RNA standard of claim 35, wherein the protein is cooperatively bound to the ribonuclease resistant RNA.

30

38. The RNA standard of claim 35, wherein the protein is gp32 of bacteriophage T4.

39. The RNA standard of claim 33, wherein the ribonuclease resistant coating is comprised of a polyamine.

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40 The RNA standard of claim 39, wherein polyamine is spermine or spermidine.

41 The RNA standard of claim 33, wherein the ribonuclease resistant coating is
5 comprised of a nucleic acid.

42 The RNA standard of claim 41, wherein the nucleic acid hybridizes to the
10 ribonuclease resistant RNA segment.

43 The RNA standard of claim 42, wherein the hybridized nucleic acid is a primer for
reverse transcriptase.

15 44 The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment
further comprises a viral Coat Protein coding sequence.

20 45 The RNA standard of claim 44, wherein the a viral Coat Protein coding sequence
codes for a bacteriophage viral Coat Protein.

25 46 The RNA standard of claim 45, wherein the bacteriophage viral Coat Protein is a
modified bacteriophage viral Coat Protein.

47 The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment
30 comprises a non-bacteriophage sequence.

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48. The RNA standard of claim 47, wherein the non-bacteriophage sequence is a viral sequence.

5 49. The RNA standard of claim 47, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of an RNA.

10 50. The RNA standard of claim 49, wherein the non-bacteriophage sequence is a sequence adapted for use in detection and/or quantification of an RNA of diagnostic value.

15 51. The RNA standard of claim 49, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.

20 52. The RNA standard of claim 51, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, or hepatitis G.

25 53. The RNA standard of claim 52, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, or HCV.

30 54. The RNA standard of claim 53, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1 or HIV-2

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55. The RNA standard of claim 54, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1.

5 56. The RNA standard of claim 55, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-1 comprises a modified HIV-1 sequence.

10 57. The RNA standard of claim 54, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-2.

15 58. The RNA standard of claim 57, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-2 comprises a modified HIV-2 sequence.

20 59. The RNA standard of claim 53, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HCV.

25 60. The RNA standard of claim 59, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HCV comprises a modified HCV sequence.

30 61. The RNA standard of claim 52, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HTLV-1.

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62. The RNA standard of claim 61, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HTLV-1 comprises a modified HTLV-1 sequence.

5

63. The RNA standard of claim 52, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HTLV-2.

10

64. The RNA standard of claim 63, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HTLV-2 comprises a modified HTLV-2 sequence.

15

65. The RNA standard of claim 51, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of a blood-borne pathogen.

20

66. The RNA standard of claim 49, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of a plasmodium, trypanosome, *Francisella tularensis*, or *Wucheria bancrofti*.

25

67. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises a bacteriophage sequence.

30

68. The RNA standard of claim 67, wherein the bacteriophage sequence is a sequence from an *E. coli* bacteriophage in serological group I, II, III, or IV.

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69 The RNA standard of claim 67, wherein the bacteriophage sequence is a sequence from an *E. coli* bacteriophage of genetic subclass A.

5 70. The RNA standard of claim 67, wherein the bacteriophage sequence is a sequence from an MS2/R17 bacteriophage.

10 71. The RNA standard of claim 67, wherein the bacteriophage sequence is a sequence from a *Pseudomonas aeruginosa* RNA bacteriophage.

15 72. The RNA standard of claim 71, wherein the bacteriophage sequence is a sequence from a *Pseudomonas aeruginosa* PRR1 or PP7 bacteriophage

73. The RNA standard of claim 67, wherein the bacteriophage sequence is a sequence from a filamentous bacteriophage.

20 74. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises at least two viral Maturase binding sites.

25 75. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises a sequence coding a Replicase protein.

30 76. The RNA standard of claim 75, wherein the sequence coding the Replicase protein codes a modified Replicase protein that is not active.

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77. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises at least two Operator sequences and a non-bacteriophage sequence.

5 78. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises an Operator sequence and a viral Coat Protein sequence.

10 79. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

15 80. The RNA standard of claim 79, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

20 81. The RNA standard of claim 80, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

25 82. The RNA standard of claim 81, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein, a non-bacteriophage sequence, and a sequence coding a Replicase protein.

30 83. The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment is coded for in a sequence of pAR-1.

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84 The RNA standard of claim 2, wherein the ribonuclease resistant RNA segment is coded for in a sequence of pAR-2.

5 85. A method of assaying for the presence of a tested nucleic acid sequence in a sample comprising:

obtaining a sample;

10 obtaining a nucleic acid standard comprising a nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid;

assaying the sample for the presence of a tested nucleic acid sequence; and

15 employing the nucleic acid segment comprising a sequence coding a standard nucleic acid as a standard in the assay.

20 86. The method of claim 85, further comprising the step of isolating a nucleic acid composition from said sample.

87. The method of claim 85, wherein said sample is a biological tissue.

25

88. The method of claim 85, wherein said sample is a blood component.

30

89. The method of claim 85, further comprising isolating the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid from a molecule that renders the nuclease resistant nucleic acid segment comprising a sequence coding a

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standard nucleic acid nuclease resistant, to obtain a nucleic acid segment comprising a sequence coding a standard nucleic acid.

5 90. The method of claim 85, further comprising admixing the sample and the nucleic acid standard comprising a nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid prior to assaying for the presence of the tested nucleic acid sequence.

10 91. The method of claim 90, further comprising isolating the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid from a molecule that renders the nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid nuclease resistant, to obtain a nucleic acid segment comprising a
15 sequence coding a standard nucleic acid.

20 92. The method of claim 91, wherein the admixing comprises admixing said sample and the nucleic acid standard prior to isolation of a nucleic acid composition from the sample and isolation of the nucleic acid segment comprising a sequence coding a standard nucleic acid so that isolation of the nucleic acid composition from the sample and the nucleic acid segment comprising a sequence coding a standard nucleic acid is performed in the same isolation procedure.

25 93. The method of claim 85, wherein the nucleic acid standard is employed as an internal nucleic acid standard.

30 94. The method of claim 85, wherein the nucleic acid standard is employed as an external nucleic acid standard.

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95 The method of claim 85, wherein the nucleic acid standard is employed as a positive control nucleic acid standard.

5 96 The method of claim 85, wherein the assay is a quantitative assay.

97 The method of claim 85, wherein the nuclease resistant nucleic acid segment is a ribonuclease resistant RNA comprising a sequence coding a standard RNA.

10

98 The method of claim 85, wherein the tested nucleic acid is an RNA.

15 99 The method of claim 85, wherein the assay step comprises PCRTM analysis.

100 The method of claim 99, wherein the assay step comprises a PCRTM analysis comprising:

20

employing a reverse transcription procedure;

amplifying a DNA to generate a PCRTM product; and

25

detecting the PCRTM product.

101 The method of claim 100, wherein the amplification step comprises co-amplification of a tested RNA PCRTM product with standard RNA PCRTM product.

30

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102. The method of claim 101, wherein the co-amplification involves the use of a single primer set adapted for amplification of both tested RNA PCR™ product and standard RNA PCR™ product from an RT-PCR™ procedure.

5

103. The method of claim 99, wherein the assay comprises comparing an amount of tested RNA PCR™ product with an amount of standard RNA PCR™ product.

10

104. The method of claim 85, wherein the nucleic acid standard is further defined as an RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA.

15

105. The method of claim 104, wherein the the ribonuclease resistant RNA segment is a chemically modified ribonuclease resistant RNA.

20

106. The method of claim 105, wherein the chemically modified ribonuclease resistant RNA is comprised of chemically modified nucleotides.

25

107. The method of claim 105, wherein the chemically modified RNA is prepared by chemical modification of a previously transcribed RNA transcript.

30

108. The RNA standard of claim 104, wherein the ribonuclease resistant RNA segment is coated with a ribonuclease resistant coating.

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109. The method of claim 108, wherein the ribonuclease resistant coating is comprised of a protein.

5 110. The method of claim 109, wherein the protein is bound in a sequence independent manner to the ribonuclease resistant RNA.

10 111. The method of claim 110, wherein the protein is MS2/R17 coat protein, HIV-1 nucleocapsid protein, gp32 of bacteriophage T4, or the regA protein of T4.

112. The method of claim 111, wherein the protein is cooperatively bound to the ribonuclease resistant RNA.

15

113. The method of claim 111, wherein the protein is gp32 of bacteriophage T4.

20 114. The method of claim 108, wherein the ribonuclease resistant coating is comprised of a polyamine.

25 115. The method of claim 114, wherein polyamine is spermine or spermidine.

116. The method of claim 108, wherein the ribonuclease resistant coating is comprised of a nucleic acid.

30

117. The method of claim 116, wherein the nucleic acid hybridizes to the ribonuclease resistant RNA segment.

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118. The method of claim 117, wherein the hybridized nucleic acid is a primer for reverse transcriptase.

5 119. The method of claim 104, wherein the recombinant RNA segment is caged in a ribonuclease resistant structure.

10 120. The method of claim 104, wherein the ribonuclease resistant RNA segment is a caged ribonuclease resistant structure.

121. The method of claim 120, wherein the ribonuclease resistant structure is comprised of lipids.

15

122. The method of claim 121, wherein the ribonuclease resistant structure is a liposome.

20

123. The method of claim 120, wherein the ribonuclease resistant structure is a synthetic microcapsule.

25

124. The method of claim 123, wherein the ribonuclease resistant structure is a polymer matrix.

30

125. The method of claim 124, wherein the polymer matrix is agarose or acrylamide.

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126. The method of claim 120, wherein the ribonuclease resistant structure is comprised of a Viral Coat protein.

5 127. The method of claim 126, wherein the viral Coat Protein is a modified viral Coat Protein.

128. The method of claim 126, wherein the ribonuclease resistant RNA segment is
10 resistant to ribonuclease when encapsidated in viral Coat Protein.

129. The method of claim 126, wherein the viral Coat Protein is a bacteriophage viral
15 Coat Protein.

130. The method of claim 126, wherein the viral Coat Protein is a viral Coat Protein of
a plant or animal virus.

20 131. The method of claim 130, wherein the bacteriophage viral Coat Protein is a viral Coat Protein of tobacco mosaic virus.

25 132. The method of claim 126, wherein the ribonuclease resistant RNA segment is bound to a viral Maturase protein.

30 133. The method of claim 132, wherein the viral Maturase protein is a modified viral Maturase protein.

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134. The method of claim 97, wherein the ribonuclease resistant RNA segment comprises an Operator coding sequence and a viral Maturase protein binding site.

5 135. The method of claim 134, wherein the viral Maturase protein binding site is a modified viral Maturase protein binding site.

10 136. The method of claim 134, wherein the Maturase protein binding site is included in a viral Maturase protein coding sequence.

15 137. The method of claim 134, wherein the ribonuclease resistant RNA segment further comprises a viral Maturase protein coding sequence.

138. The method of claim 137, wherein the Maturase protein coding sequence codes a modified viral Maturase protein.

20

139. The method of claim 97, wherein the ribonuclease resistant RNA segment comprises a viral Coat Protein coding sequence

25 140. The method of claim 139, wherein the viral Coat Protein coding sequence codes for a bacteriophage viral Coat Protein.

30 141. The method of claim 139, wherein the bacteriophage viral Coat Protein is a modified bacteriophage viral Coat Protein.

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142. The method of claim 97, wherein the sequence coding a standard RNA comprises a non-bacteriophage sequence.

5 143. The method of claim 142, wherein the non-bacteriophage sequence is a viral sequence.

10 144. The method of claim 142, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of an RNA.

15 145. The method of claim 144, wherein the non-bacteriophage sequence is a sequence adapted for use in detection and/or quantification of an RNA of diagnostic value.

20 146. The method of claim 144, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.

25 147. The method of claim 146, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, or HCV.

30 148. The method of claim 147, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, or HCV comprises a modified HIV-1, HIV-2, or HCV sequence.

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149. The method of claim 147, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1.

5 150. The method of claim 149, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-1 comprises a modified HIV-1 sequence.

10 151. The method of claim 146, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of a blood-borne pathogen.

15 152. The method of claim 97, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

20 153. The method of claim 97, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

25 154. The method of claim 97, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

30 155. The method of claim 97, wherein the recombinant RNA segment comprises a sequence coding a Replicase protein.

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156. The method of claim 97, wherein the recombinant RNA segment is coded for in a sequence of pAR-1 or pAR-2.

5 157. A ribonuclease resistant RNA segment comprising an Operator coding sequence and a viral Maturase protein binding site.

10 158. The ribonuclease resistant RNA segment of claim 157, wherein the Maturase protein binding site is included in a viral Maturase protein coding sequence.

15 159. The ribonuclease resistant RNA segment of claim 157, wherein the Maturase protein binding site is a modified Maturase protein binding site.

20 160. The ribonuclease resistant RNA segment of claim 158, further comprising a viral Maturase protein coding sequence.

161. The ribonuclease resistant RNA segment of claim 160, wherein the Maturase protein coding sequence codes a modified viral Maturase protein.

25 162. The ribonuclease resistant RNA segment of claim 157, further comprising a viral Coat Protein coding sequence.

30 163. The ribonuclease resistant RNA segment of claim 162, wherein the a viral Coat Protein coding sequence codes for a bacteriophage viral Coat Protein

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164. The ribonuclease resistant RNA segment of claim 163, wherein the bacteriophage viral Coat Protein is a modified bacteriophage viral Coat Protein.

5 165. The ribonuclease resistant RNA segment of claim 157, further comprising a non-bacteriophage sequence.

10 166. The ribonuclease resistant RNA segment of claim 165, wherein the non-bacteriophage sequence is a viral sequence.

15 167. The ribonuclease resistant RNA segment of claim 165, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard for detection of a disease.

20 168. The ribonuclease resistant RNA segment of claim 165, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.

25 169. The ribonuclease resistant RNA segment of claim 168, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, or hepatitis G.

30 170. The ribonuclease resistant RNA segment of claim 169, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, or HCV.

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171. The ribonuclease resistant RNA segment of claim 170, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1 or HIV-2.

5

172. The ribonuclease resistant RNA segment of claim 171, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-1.

10

173. The ribonuclease resistant RNA segment of claim 172, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-1 comprises a modified HIV-1 sequence.

15

174. The ribonuclease resistant RNA segment of claim 171, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HIV-2.

20

175. The ribonuclease resistant RNA segment of claim 174, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HIV-2 comprises a modified HIV-2 sequence.

25

176. The ribonuclease resistant RNA segment of claim 170, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HCV.

30

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177. The ribonuclease resistant RNA segment of claim 176, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HCV comprises a modified HCV sequence.

5

178. The ribonuclease resistant RNA segment of claim 169, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HTLV-1.

10

179. The ribonuclease resistant RNA segment of claim 178, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HTLV-1 comprises a modified HTLV-1 sequence.

15

180. The ribonuclease resistant RNA segment of claim 169, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of HTLV-2.

20

181. The ribonuclease resistant RNA segment of claim 180, wherein the non-bacteriophage sequence adapted for use as a standard in detection and/or quantification of HTLV-2 comprises a modified HTLV-2 sequence.

25

182. The ribonuclease resistant RNA segment of claim 168, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or quantification of a blood-borne pathogen.

30

183. The ribonuclease resistant RNA segment of claim 182, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard in detection and/or

quantification of a plasmodium, trypanosome, *Francisella tularensis*, or *Wucheria bancrofti*.

5 184. The ribonuclease resistant RNA segment of claim 157, further comprising a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

10 185. The ribonuclease resistant RNA segment of claim 184, further comprising a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

15 186. The ribonuclease resistant RNA segment of claim 157, further comprising at least two viral Maturase binding sites.

20 187. The ribonuclease resistant RNA segment of claim 157, further comprising a sequence coding an Operator sequence, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

25 188. A DNA vector adapted for use in the synthesis of a RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA encapsidated in viral Coat Protein.

30 189. The vector of claim 188, wherein the vector comprises a sequence coding an Operator sequence and a sequence coding a viral Maturase binding site.

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190. The vector of claim 189, wherein the multiple cloning site is downstream of the sequence encoding a viral Maturase binding site

5 191. The vector of claim 189, further comprising a sequence coding a viral Maturase protein.

10 192. The vector of claim 191, wherein the sequence coding the viral Maturase binding site is comprised within the sequence coding the viral Maturase protein.

15 193. The vector of claim 189, further comprising a sequence coding a viral Coat Protein gene.

20 194. The vector of claim 193, further comprising a sequence coding a viral Coat Protein gene, an Operator sequence, and a multiple cloning site.

25 195. The vector of claim 188, further comprising a DNA sequence coding a non-bacteriophage sequence.

30 196. The vector of claim 195, wherein the non-bacteriophage sequence is a viral non-bacteriophage sequence.

197. The vector of claim 196, wherein the non-bacteriophage sequence is adapted for use as a standard for detection and/or quantification of an RNA of diagnostic value.

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198. A collection tube containing a nuclease resistant nucleic acid standard comprising comprising a sequence coding a standard nucleic acid encapsidated in viral Coat Protein.

5 199. The collection tube of claim 198, wherein the tube is adapted for use in collection of a body fluid.

10 200. The collection tube of claim 199, wherein the body fluid is blood, urine, or cerebrospinal fluid.

15 201. A method of making an RNA standard comprising a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA encapsidated in viral Coat Protein comprising:

obtaining a vector comprising a DNA sequence coding a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence;

20

transfecting the vector into a cell;

25 providing a viral Coat Protein;

providing a viral Maturase protein; and

30 culturing the cell under conditions allowing for transcription of the ribonuclease resistant RNA segment and encapsidation of the ribonuclease resistant RNA segment in viral Coat Protein.

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202. The method of claim 201, wherein the step of providing the viral Coat Protein comprises:

obtaining a nucleic acid segment coding a viral Coat Protein;

5 transfecting the nucleic acid segment coding the viral Coat Protein into the cell;
 and

 culturing the cell under conditions allowing for expression of the viral Coat
 Protein.

10

203. The method of claim 202, wherein the nucleic acid segment coding the viral Coat Protein is a DNA sequence comprised in the vector comprising the DNA sequence coding the ribonuclease resistant RNA segment.

15

204. The method of claim 203, wherein the DNA sequence coding the viral Coat Protein is located *cis* to the DNA sequence coding the ribonuclease resistant RNA segment.

20

205. The method of claim 203, wherein the DNA sequence coding the viral Coat Protein is located in the DNA sequence coding the ribonuclease resistant RNA segment.

25

206. The method of claim 203, wherein the DNA sequence coding the viral Coat Protein is located *trans* to the DNA sequence coding the ribonuclease resistant RNA segment.

30

207. The method of claim 201, wherein the step of providing the viral Maturase protein comprises:

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obtaining a nucleic acid segment coding a viral Maturase protein;
transfecting the nucleic acid segment coding the viral Maturase protein into the
cell; and

5 culturing the cell under conditions allowing for expression of the viral Maturase
protein.

208. The method of claim 207, wherein the nucleic acid segment coding the viral
10 Maturase protein is a DNA sequence comprised in the vector comprising the DNA
sequence coding the ribonuclease resistant RNA segment.

209. The method of claim 207, wherein the DNA sequence coding the viral Maturase
15 protein is located *cis* to the DNA sequence coding the ribonuclease resistant RNA
segment.

210. The method of claim 207, wherein the DNA sequence coding the viral Maturase
20 protein is located in the DNA sequence coding the ribonuclease resistant RNA segment.

211. The method of claim 207, wherein the DNA sequence coding the viral Maturase
protein is located *trans* to the DNA sequence coding the ribonuclease resistant RNA
25 segment.

212. The method of claim 201, wherein the non-bacteriophage sequence is downstream
of the sequence encoding the viral Maturase binding site.

30

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213. The method of claim 201, wherein the ribonuclease resistant RNA segment further comprises a sequence coding a viral Maturase protein.

5 214. The method of claim 213, wherein the sequence coding the viral Maturase binding site is comprised within the sequence coding the viral Maturase protein.

10 215. The method of claim 201, wherein the ribonuclease resistant RNA segment further comprises a sequence coding a viral Coat Protein gene.

15 216. The method of claim 201, wherein the non-bacteriophage sequence is a viral non-bacteriophage sequence.

20 217. The method of claim 216, wherein the non-bacteriophage sequence is adapted for use as a standard RNA.

218. The method of claim 217, wherein the non-bacteriophage sequence is adapted for use as a standard RNA in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.

25 219. The method of claim 218, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard RNA in detection and/or quantification of HIV-1.

30 220. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises at least two viral Maturase binding sites.

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221. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises a sequence coding a Replicase protein.

5 222. The method of claim 221, wherein the sequence coding the Replicase protein codes a modified Replicase protein.

10 223. The method of claim 201, wherein the ribonuclease resistant RNA segment consists essentially of an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

15 224. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises at least two Operator sequences and a non-bacteriophage sequence.

20 225. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises at least two Operator sequences and a sequence coding a viral Coat Protein.

25 226. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

30 227. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

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228. The method of claim 201, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein, a non-bacteriophage sequence, and a sequence coding a Replicase protein.

5

229. The method of claim 201, wherein the ribonuclease resistant RNA segment is coded for in a sequence of pAR-1.

10

230. The method of claim 201, wherein the ribonuclease resistant RNA segment is coded for in a sequence of pAR-2.

15

231. The method of claim 201, wherein the cell is an *E. coli* cell.

232. A method of making a nucleic acid standard comprising a nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid encapsidated in viral Coat Protein comprising:

20

obtaining a vector comprising a nucleic acid sequence coding a nuclease resistant nucleic acid segment comprising a sequence coding a standard nucleic acid comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence;

25

transfecting the vector into a cell;

providing a viral Coat Protein;

30

providing a viral Maturase protein; and

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culturing the cell under conditions allowing for transcription of the recombinant nucleic acid segment and encapsidation of the recombinant nucleic acid segment in viral Coat Protein.

5

233. The method of claim 232, wherein the nuclease resistant nucleic acid segment is RNA.

10

234. The method of claim 232, wherein the nuclease resistant nucleic acid segment is DNA.

15

235. A method of making an RNA standard comprising a ribonuclease resistant RNA segment encapsidated in viral Coat Protein comprising:

20

obtaining a vector comprising a DNA sequence coding a recombinant RNA segment comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence;

transfecting the vector into a cell;

25

obtaining a DNA segment coding a viral Coat Protein and transfecting the nucleic acid segment coding the viral Coat Protein into the cell;

obtaining a DNA segment coding a viral Maturase protein and transfecting the nucleic acid segment coding the viral Maturase protein into the cell; and

30

culturing the cell under conditions allowing for transcription of the recombinant RNA segment, expression of the viral Coat Protein and the viral Maturase protein, and encapsidation of the recombinant RNA segment in viral Coat

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Protein to obtain a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA.

5 236. The method of claim 235, wherein the DNA segment coding the viral Coat Protein is comprised in the vector comprising the DNA sequence coding the recombinant RNA segment.

10 237. The method of claim 236, wherein the DNA sequence coding the viral coat protein is located *cis* to the DNA sequence coding the recombinant RNA segment.

15 238. The method of claim 236, wherein the DNA sequence coding the viral Coat Protein is located in the DNA sequence coding the recombinant RNA segment.

20 239. The method of claim 236, wherein the DNA sequence coding the viral Coat Protein is located *trans* to the DNA sequence coding the recombinant RNA segment.

25 240. The method of claim 235, wherein the DNA segment coding the viral Maturase protein is comprised in the vector comprising the DNA sequence coding the recombinant RNA segment.

30 241. The method of claim 240, wherein the DNA sequence coding the viral Maturase protein is located *cis* to the DNA sequence coding the recombinant RNA segment.

242. The method of claim 240, wherein the DNA sequence coding the viral Maturase protein is located in the DNA sequence coding the recombinant RNA segment.

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243. The method of claim 240, wherein the DNA sequence coding the viral Maturase protein is located *trans* to the DNA sequence coding the recombinant RNA segment.

5 244. The method of claim 235, wherein the non-bacteriophage sequence is downstream of the sequence encoding the viral Maturase binding site.

10 245. The method of claim 235, wherein the recombinant RNA segment further comprises a sequence coding a viral Maturase protein.

15 246. The method of claim 245, wherein the sequence coding the viral Maturase binding site is comprised within the sequence coding the viral Maturase protein.

20 247. The method of claim 235, wherein the recombinant RNA segment further comprises a sequence coding a viral Coat Protein gene.

248. The method of claim 235, wherein the non-bacteriophage sequence is a viral non-bacteriophage sequence.

25 249. The method of claim 235, wherein the non-bacteriophage sequence is adapted for use as a standard RNA.

30 250. The method of claim 249, wherein the non-bacteriophage sequence is adapted for use as a standard RNA in detection and/or quantification of HIV-1, HIV-2, HCV, HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.

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251. The method of claim 250, wherein the non-bacteriophage sequence is a sequence adapted for use as a standard RNA in detection and/or quantification of HIV-1.

5 252. The method of claim 235, wherein the recombinant RNA segment comprises at least two viral Maturase binding sites.

10 253. The method of claim 235, wherein the recombinant RNA segment comprises a sequence coding a Replicase protein.

254. The method of claim 253, wherein the sequence coding the Replicase protein codes a modified Replicase protein.

15 255. The method of claim 235, wherein the recombinant RNA segment consists essentially of an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

20 256. The method of claim 235, wherein the recombinant RNA segment comprises an Operator sequence and a sequence coding a viral Coat Protein.

25 257. The method of claim 235, wherein the recombinant RNA segment comprises at least two Operator sequences and a non-bacteriophage sequence.

30 258. The method of claim 235, wherein the recombinant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

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259. The method of claim 235, wherein the recombinant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

5

260. The method of claim 259, wherein the recombinant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein, a non-bacteriophage sequence, and a sequence coding a Replicase protein.

10

261. The method of claim 235, wherein the recombinant RNA segment is coded for in a sequence of pAR-1.

15

262. The method of claim 235, wherein the recombinant RNA segment is coded for in a sequence of pAR-2.

20

263. The method of claim 235, wherein the cell is an *E. coli* cell.

264. A method of making RNA *in vivo* comprising:

25

obtaining a vector comprising a DNA sequence coding a ribonuclease resistant RNA segment comprising a sequence coding a standard RNA comprising a sequence coding an Operator sequence, a sequence coding a viral Maturase binding site, and a non-bacteriophage sequence;

30

transfecting the vector into a cell;

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obtaining a DNA segment coding a viral Coat Protein and transfecting the nucleic acid segment coding the viral Coat Protein into the cell;
obtaining a DNA segment coding a viral Maturase protein and transfecting the nucleic acid segment coding the viral Maturase protein into the cell; and
5 culturing the cell under conditions allowing for transcription of the ribonuclease resistant RNA segment, expression of the viral Coat Protein and the viral Maturase protein, and encapsidation of the ribonuclease resistant RNA segment in viral Coat Protein.

10 265. The method of claim 264, further comprising the step of isolating the ribonuclease resistant RNA segment from the Coat Protein.

15 266. The method of claim 264, further comprising the step of treating the isolated RNA segment to obtain an RNA segment comprising the non-bacteriophage sequence.

20 267. The method of claim 264, further comprising the step of treating the isolated RNA segment to obtain an RNA segment consisting essentially of the non-bacteriophage sequence.

25 268. The method of claim 264, further comprising the step of treating the isolated RNA segment to obtain an RNA segment consisting of the non-bacteriophage sequence.

30 269. The method of claim 264, wherein the DNA segment coding the viral Coat Protein is comprised in the vector comprising the DNA sequence coding the ribonuclease resistant RNA segment.

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270. The method of claim 269, wherein the DNA sequence coding the viral coat protein is located *cis* to the DNA sequence coding the ribonuclease resistant RNA segment.

5

271. The method of claim 269, wherein the DNA sequence coding the viral Coat Protein is located in the DNA sequence coding the ribonuclease resistant RNA segment.

10

272. The method of claim 269, wherein the DNA sequence coding the viral Coat Protein is located *trans* to the DNA sequence coding the ribonuclease resistant RNA segment.

15

273. The method of claim 264, wherein the DNA segment coding the viral Maturase protein is comprised in the vector comprising the DNA sequence coding the ribonuclease resistant RNA segment.

20

274. The method of claim 273, wherein the DNA sequence coding the viral Maturase protein is located *cis* to the DNA sequence coding the ribonuclease resistant RNA segment.

25

275. The method of claim 273, wherein the DNA sequence coding the viral Maturase protein is located in the DNA sequence coding the ribonuclease resistant RNA segment.

30

276. The method of claim 273, wherein the DNA sequence coding the viral Maturase protein is located *trans* to the DNA sequence coding the ribonuclease resistant RNA segment.

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277. The method of claim 276, wherein the non-bacteriophage sequence is downstream of the sequence encoding the viral Maturase binding site.

5 278. The method of claim 264, wherein the recombinant RNA segment further comprises a sequence coding a viral Maturase protein.

279. The method of claim 278, wherein the sequence coding the viral Maturase binding site is comprised within the sequence coding the viral Maturase protein.
10

280. The method of claim 264, wherein the ribonuclease resistant RNA segment further comprises a sequence coding a viral Coat Protein gene.
15

281. The method of claim 264, wherein the non-bacteriophage sequence is a viral non-bacteriophage sequence.
20

282. The method of claim 264, wherein the ribonuclease resistant RNA segment consists essentially of an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.
25

283. The method of claim 264, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.
30

284. The method of claim 264, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence

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coding a viral Maturase protein, a sequence coding a viral Coat Protein and a non-bacteriophage sequence.

5 285. The method of claim 264, wherein the ribonuclease resistant RNA segment comprises an Operator sequence, at least two viral Maturase binding sites, a sequence coding a viral Maturase protein, a sequence coding a viral Coat Protein, a non-bacteriophage sequence, and a sequence coding a Replicase protein.

10

286. The method of claim 264, wherein the ribonuclease resistant RNA segment comprises at least two viral Maturase binding sites.

15

287. The method of claim 264, wherein the ribonuclease resistant RNA segment comprises a sequence coding a Replicase protein.

20

288. The method of claim 287, wherein the sequence coding the Replicase protein codes a modified Replicase protein.

25

289. A method of encapsidating a recombinant RNA segment in viral Coat Protein *in vitro* comprising:

obtaining a recombinant RNA segment;

obtaining viral Coat Protein; and

30

placing the recombinant RNA segment and the viral Coat Protein together under conditions causing the recombinant RNA segment to become encapsidated in the viral Coat Protein.

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290. The method of claim 289, wherein the viral Coat Protein is a bacteriophage viral Coat Protein.

5 291. The method of claim 290, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage of genetic subclass A or B.

292. The method of claim 291, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage of genetic subclass A.
10

293. The method of claim 290, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage in serological group I, II, II, or IV.
15

294. The method of claim 293, wherein the bacteriophage viral Coat Protein is of an *E. coli* bacteriophage in serological group I.
20

295. The method of claim 290, wherein the bacteriophage viral Coat Protein is of an MS2/R17 bacteriophage.

25 296. The method of claim 290, wherein the bacteriophage viral Coat Protein is of a *Pseudomonas aeruginosa* RNA bacteriophage.

297. The method of claim 296, wherein the bacteriophage viral Coat Protein is of a *Pseudomonas aeruginosa* PRR1 or PP7 bacteriophage
30

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298. The method of claim 290, wherein the bacteriophage viral Coat Protein is of a filamentous bacteriophage.

5 299. The method of claim 289, wherein the viral Coat Protein is a viral Coat Protein of a plant or animal virus.

10 300. The method of claim 299, wherein the viral Coat Protein is a viral Coat Protein of a tobacco mosaic virus, a feline immunodeficiency virus, or a Rous sarcoma virus.

15 301. The method of claim 289, further comprising a provision of viral Maturase protein.

20 302. The method of claim 301, wherein the viral Maturase protein is a modified viral Maturase protein.

25 303. The method of claim 289, wherein the recombinant RNA segment comprises an Operator coding sequence and a viral Maturase protein binding site.

30 304. The method of claim 303, wherein the recombinant RNA segment further comprises a viral Maturase protein coding sequence.

305. The method of claim 303, wherein the recombinant RNA segment further comprises a viral Coat Protein coding sequence

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306. The method of claim 290, wherein the recombinant RNA segment comprises a bacteriophage sequence from an RNA bacteriophage and a non-bacteriophage sequence.

5 307. The method of claim 306, wherein the non-bacteriophage sequence is a viral sequence.

308. The method of claim 306, wherein the non-bacteriophage sequence is a sequence
10 adapted for use as a standard in detection and/or quantification of an RNA.

309. The method of claim 308, wherein the non-bacteriophage sequence is a sequence
15 adapted for use in detection and/or quantification of an RNA of diagnostic value.

310. The method of claim 308, wherein the non-bacteriophage sequence is a sequence
adapted for use as a standard in detection and/or quantification of HIV-1, HIV-2, HCV,
HTLV-1, HTLV-2, hepatitis G, an enterovirus, or a blood-borne pathogen.
20

311. The method of claim 310, wherein the non-bacteriophage sequence is a sequence
adapted for use as a standard in detection and/or quantification of HIV-1.

25 312. The method of claim 311, wherein the non-bacteriophage sequence adapted for
use as a standard in detection and/or quantification of HIV-1 comprises a modified HIV-1
sequence.

30 313. The method of claim 306, wherein the bacteriophage sequence is a sequence from
an *E. coli* bacteriophage of genetic subclass A.

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314. The method of claim 290, wherein the recombinant RNA segment comprises at least two viral Maturase binding sites.

5 315. The method of claim 290, wherein the recombinant RNA segment comprises a sequence coding a Replicase protein.

316. The method of claim 315, wherein the sequence coding the Replicase protein codes a modified Replicase protein that is not active.

10

317. The method of claim 290, wherein the recombinant RNA segment comprises at least two Operator sequences and a non-bacteriophage sequence..

15

318. The method of claim 290, wherein the recombinant RNA segment comprises an Operator sequence, a sequence coding a viral Maturase protein, and a non-bacteriophage sequence.

20

319. A method of delivering RNA to cells *in vitro* or *in vivo* comprising:

obtaining an Armored RNA™ comprising a ribonuclease resistant RNA segment encapsidated in viral Coat Protein;

25

placing the Armored™ RNA culture with a cell; and

culturing the cell under conditions that cause the Armored RNA™ to be taken into the cell.

30

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320. The method of claim 319, wherein the Armored RNA™ comprises a bacteriophage protein that has been modified to facilitate delivery of RNA to a cell.

- 5 321. The method of claim 320, wherein the modified bacteriophage protein is a viral Coat Protein or a Maturase protein.

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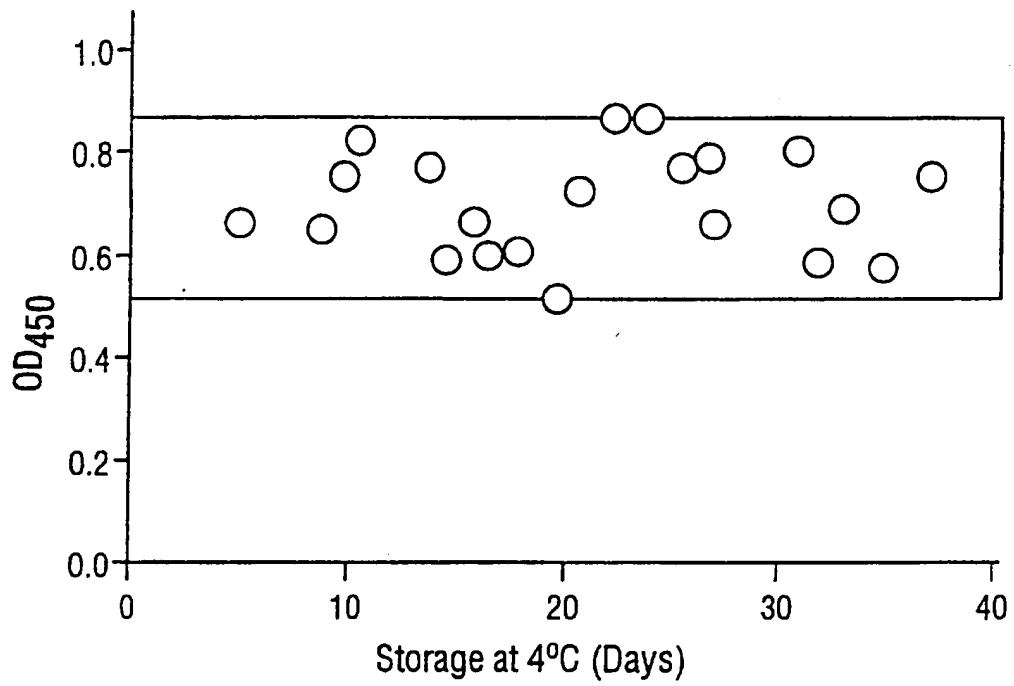


FIG. 1

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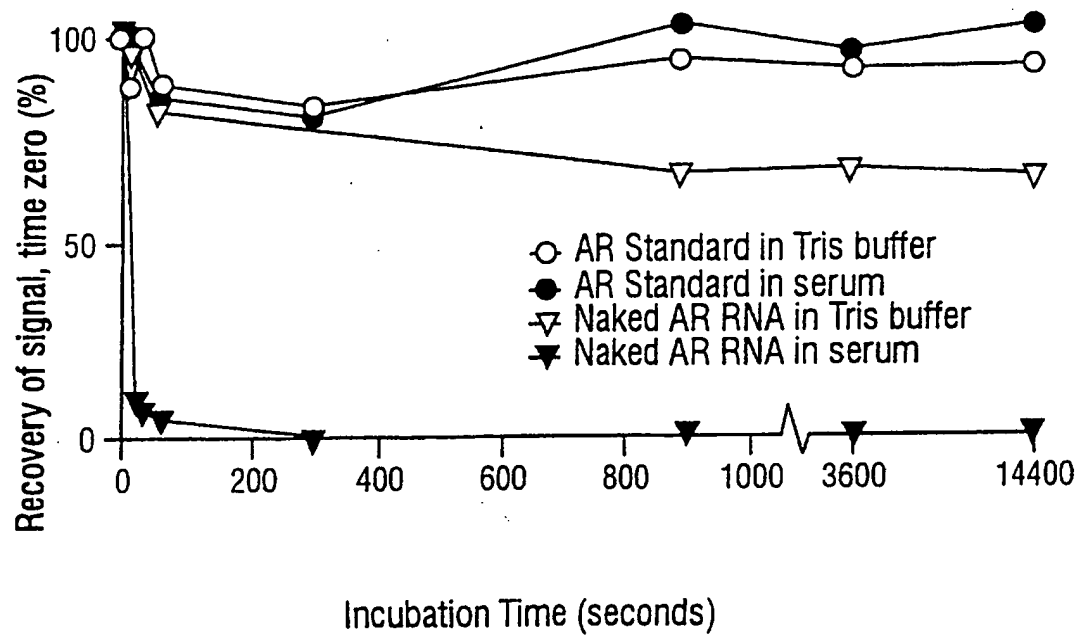


FIG. 2

INTERNATIONAL SEARCH REPORT

Internat. Application No

PCT/US 97/12551

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/40 C12N15/48 C12N15/51 C12N15/10 C12N15/88 C12N7/04 C12Q1/68 C12Q1/70 C12P19/34			
According to International Patent Classification (IPC) or to both national classification and IPC			
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C12Q C12P			
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched			
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)			
C. DOCUMENTS CONSIDERED TO BE RELEVANT			
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.	
X	WO 94 28171 A (PNA DIAGNOSTICS A S ;STANLEY CHRISTOPHER JOHN (GB)) 8 December 1994 see the whole document ---	1,85-93, 96,99, 100	
A	REISCHL U ET AL: "QUANTITATIVE PCR A SURVEY OF THE PRESENT TECHNOLOGY" MOLECULAR BIOTECHNOLOGY, vol. 3, pages 55-71, XP000600241 see the whole document ---	1-321	
A	WO 95 02067 A (AKZO NOBEL NV ;BEUNINGEN MARINUS GERARDUS JOH (NL); KIEVITS TIM (N) 19 January 1995 see the whole document ---	1-321	
-/-			
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.			
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "Z" document member of the same patent family			
Date of the actual completion of the international search		Date of mailing of the international search report	
13 October 1997		21.10.97	
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl Fax: (+31-70) 340-3016		Authorized officer Hornig, H	

INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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A	WO 95 14109 A (U S DEPARTMENT OF THE ARMY) 26 May 1995 see the whole document ---	1-321
A	WO 95 34684 A (UNIV GEORGETOWN) 21 December 1995 see the whole document ---	1-321
A	EP 0 525 882 A (AKZO NV) 3 February 1993 see the whole document ---	1-321
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A	N.J. STONEHOUSE AND P.G. STOCKLEY: "Effect of amino acid substitution on the thermal stability of MS2 capsids lacking genomic RNA" FEBS LETTERS, vol. 334, no. 3, November 1993, ELSEVIER, AMSTERDAM, NL, pages 355-389, XP002043379 cited in the application see the whole document ---	1-321
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A	P.G. STOCKLEY ET AL.: "Molecular mechanism of RNA phage morphogenesis" INT. J. BIOCHEM., vol. 26, no. 10/11, 1994, PERGAMON PRESS PLC, GREAT BRITAIN, pages 1249-1260, XP002043381 cited in the application see the whole document ---	1-321
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A	O. HEIDENREICH ET AL.: "Chemically modified RNA: approaches and applications" FASEB JOURNAL, vol. 7, 1993, FASEB, BATHESDA, MD, US, pages 90-96, XP002043382 cited in the application see the whole document	1-321

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